

**EFFICACY OF VACCINES AGAINST VARIANT INFECTIOUS BURSAL DISEASE
VIRUSES TO CONTROL IMMUNOSUPPRESSION IN THE BROILER
CHICKEN INDUSTRY IN CANADA**

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By

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ABSTRACT

Infectious bursal disease (IBD) is a highly immunosuppressive viral disease in chickens. Currently, the antigenically variant strains of infectious bursal disease virus (vIBDV) are the most prevalent strains of IBDV circulating in Canada.

The main objective of the of my research is to investigate the protective efficacy of vaccines against the immunosuppressive effects of IBDV currently circulating in Canada. In the second chapter, we conducted a series of controlled challenge experiments in chickens using vIBDV-SK09, which represents one of the most prevalent field strains of vIBDV in Canada. In this study, we challenged specific pathogen free (SPF) leghorns, maternal antibody (MAb) free broilers, and MAb carrying broilers. Our results revealed that vIBDV-SK09 is pathogenic and capable of breaking through MABs.

In the third chapter, we found that vIBDV-SK09 can cause immunosuppression, resulting in significantly higher mortality and disease severity in pre-exposed chickens upon challenge with a virulent strain of *Escherichia coli*. In the fourth chapter, we evaluated two commercial broiler vaccines, recombinant herpes virus of turkey (rHVT)-IBDV and modified live vaccine (MLV), and found that both vaccines failed to confer complete protection against vIBDV-SK09 infection in broilers. However, the MLV but not the rHVT-IBDV vaccine was able to delay vIBDV-SK09 pathogenesis. We also revealed the potential of immunosuppression by rHVT-IBD that allowed early replication of challenged IBDV, thus increasing the viral load in the bursa. In the fifth chapter, we tested five circulating strains of vIBDVs (SK09, SK10, SK11, SK12, and SK13) as potential broiler-breeder vaccine candidates. Progeny challenge using SK09 as challenge virus demonstrated homologous and heterologous protection by SK09 as a vaccine candidate.

In conclusion, the overall findings in this thesis demonstrate that vIBDV-SK09 is pathogenic, not amenable to current commercial IBD vaccines, and can cause immunosuppression which in turn may increase the susceptibility of birds to secondary infections such as *E. coli*. Our data suggest that antigenically relevant vaccine candidate(s) such as vIBDV-SK09 may be useful in controlling IBDV infections in the Canadian broiler chicken industry. Although, we have demonstrated the efficacy of vIBDV-SK09 under laboratory conditions further studies are needed under field situations.

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TO MY LOVING PARENTS....
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LIST OF ABBREVIATIONS

ACU	animal care unit
AGPT	agar gel precipitation test
BBW	bursal weight to body weight percentage
BF	bursa of Fabricius
CAM	chorioallantoic membrane
CCS	cumulative clinical score
CD	cluster of differentiation
CEF	chicken embryo fibroblast
CFU	colony forming unit
cIBDV	classical strain of IBDV
Ct	cycle threshold
Del-E	Delaware-E
dsRNA	double stranded ribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EID ₅₀	embryo infective dose 50
ELISA	enzyme linked immunosorbent assay
FAT	fluorescence antibody technique
GM	geometric mean
H&E	hematoxylin and eosin
IBA	infectious bursal disease agent
IBD	infectious bursal disease
IBDV	infectious bursal disease virus
IBV	infectious bronchitis virus
Icx	immune complex
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNO	inductible nitrogen oxide
MAb	maternally-derived antibodies
MLV	modified live vaccine

mRNA	messenger ribonucleic acid
NC-L	North Carolina-like
NTC	no template control
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKR	protein kinase R
PP	poly protein
pi	post infection
pVP2	precursor viral protein 2
RdRp	RNA-dependent RNA polymerase
RE	restriction enzyme
RELp	restriction fragment length polymorphism
rHVT	recombinant herpes virus of turkey
RNP	ribonucleoprotein complexes
RR	relative risk
RT	reverse transcriptase
rVP2	recombinant viral protein 2
SD	standard deviation
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssRNA	single stranded ribonucleic acid
SPF	specific pathogen free
SVP	subviral particles
TCID ₅₀	tissue culture infective dose
TNF	tumor necrosis factor
TGF	transforming growth factor
vIBDV	variant strain of IBDV
VN	virus neutralization
VP	viral protein
VPg	genomic linked viral protein

vvIBDV

very virulent IBDV

CHAPTER 1 : INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Infectious bursal disease virus (IBDV) causes infectious bursal disease (IBD), also called Gumboro disease, affecting young chickens 3-6 weeks of age. IBDV is highly contagious and is very resistant to most disinfectants and environmental factors. It is hard to remove from contaminated barns, where it can persist for months in premises, feed, water, and droppings. IBDV replicates in the bursal tissue causing severe apoptosis of lymphoid cells in the bursa of Fabricius (BF) leading to immunosuppression in chicken. IBD is one the most important immunosuppressive diseases that creates grave economic problems for the poultry industry worldwide. The economic impacts of IBDV are manifold that include not only the direct losses due to morbidity and mortality, but the immunosuppression induced by this virus exacerbating infections with other pathogens and causing vaccination failures leading to secondary infection. IBDV is classified into two distinct serotypes (i.e. serotype I and II). Serotype I viruses are pathogenic to chickens and are further classified into classic, variant, and highly virulent strains; whereas serotype II viruses, isolated from turkeys, are apathogenic to chickens. IBDV in chickens has been controlled by a vaccination strategy to maximize maternal antibodies (MAb) against IBDV by hyper-immunization of breeder parents. There has been a significant rise in IBDV infection-associated production losses in Canadian broiler chicken farms and clinical signs associated with respiratory and enteric diseases and vaccine failures. Overall, tremendous progress has been made in recent years in the field which led to a better understanding of this virus, development of vaccines and diagnostic tools against this disease.

1.2 History

In 1957, Albert S. Cosgrove recognized a new disease syndrome in chicken at a poultry farm (Bunting Farm) located in Gumboro, Delaware, USA (1). The clinical signs observed were diarrhea, ruffled feathers, trembling and depression. The primary lesions were enlarged kidneys

with pronounced tubule degeneration, edematous BF and hemorrhagic thigh muscles. This disease was termed as “Gumboro Disease” based on the geographical location of its first outbreak and also named as “Avian Nephrosis” due to the involvement of kidney lesions (1). In 1962, Winterfeid and Hitchner recognized the viral nature of the infection and termed this disease as “Nephritis and Nephrosis Syndrome” (2). Two variant strains (Gray and Holte) of infectious bronchitis virus (IBV) were implicated as the causative agents of this disease syndrome (2). Given the similarity in the renal lesions as observed in IBV (Gray strain) and Avian Nephrosis, the disease was termed as “Nephritis and Nephrosis Syndrome”. Subsequent investigation reported that the virus causing Gumboro disease was able to infect birds already immune to Gray virus and inflicted changes in the cloacal bursa (3). Winterfield isolated the infectious agent in embryonated eggs and named it as infectious bursal disease agent (IBA) (4). By the late 1960s, pathogenesis of IBA was further explained (5, 6) and it was concluded that the etiological agent is indeed a virus which is highly resistant to extreme conditions of pH and temperature and a wide range of disinfectants (7, 8). Edgar in 1961 named this disease syndrome as IBD (3). Infectivity of IBDV for embryonated eggs was successfully shown by Hitchner, S.B. in 1970 (8). However in some of his initial studies, a variability in the degree of virus multiplication and certain eggs would inhibit the virus growth was observed suggesting that parental antibodies from immune dams may have inhibited the virus (8). Hitchner further demonstrated the ability of maternally transferred passive antibodies in protecting young birds at an early age from IBDV infection (9). Subsequently, the taxonomic position of the virus has been discussed and suggested to place in a new taxonomic group (10).

In 1979 a Canadian scientist, Peter Dobos, illustrated the biophysical and biochemical properties of IBDV and five other naked, icosahedral, bi-segmented, RNA viruses (infectious pancreatic necrosis virus, Tellina virus, oyster virus of bivalve molluscs and drosophila X virus) and suggested that these viruses could not be placed into any previously recognized group but should be placed in a new taxonomic category called collectively “Bi-RNAviruses”. The suffix bi- signifies double-strandedness, as well as the bisegmented nature of the virus genome, whereas “RNA” indicates the type of the viral nucleic acid (11). The first report of describing the viral proteins (VPs) was published by Nick et al., in 1976. They have analyzed the polypeptides of the purified virus and recognized 4 major VPs 1-4 (10). In 1979, it was further discovered that IBDV genome consists of two segments of double-stranded RNA namely, A and B (12).

1.3 *Birnaviridae* taxonomy

Following the discovery of IBD, there have been several changes in the taxonomic placement of the causative agent, IBDV. The virus has been placed in a variety of families, including *Picornaviridae* family (13) and *Reoviridae* family (14). A better understanding and knowledge of morphological and physicochemical properties of the IBDV led to the current taxonomic nomenclature that placed IBDV in genus and family *Birnaviridae* (11) and order not assigned (www.ictvonline.org). The family *Birnaviridae* is composed of 4 principal genus; Aquabirnavirus, Avibirnavirus, Blosnavirus and Entomobirnavirus. IBDV is categorized under the genus Avibirnavirus (15), whereas, infectious pancreatic necrosis virus, blotched snakehead virus and drosophila X virus are classified under the genus Aquabirnavirus, Blosnavirus and Entomobirnavirus, respectively (www.ictvonline.org). Besides the numerous reports of IBDV infection in chickens, IBDV has also been isolated from turkey (16), ducks (17), guinea fowl (18) and penguins (19). All viruses of the *Birnaviridae* family contain two segments of double-stranded RNA (dsRNA) surrounded by a single protein capsid with icosahedral symmetry (11).

1.4 Genome organization of *Birnaviridae*

IBDV has a bi-segmented dsRNA genome (20). The genome is composed of 2 segments; larger segment A and smaller segment B (85) with a molecular weight of 2.2×10^6 and 1.9×10^6 Da, respectively (21). In 1995, Mundt and Muller reported the first complete nucleotide sequences of the IBDV genome (segment A and segment B) (22). Segment A contains two partly overlapping open reading frames (ORF) and large ORF encodes a polyprotein (10^7 kDa, NH₃-pVP2-VP4-VP3-COOH) which is cleaved by the proteolytic activity of VP4 yielding 3 polypeptides, the precursor VP2 (pVP2), VP3, and a serine protease VP4 (23, 24). The precursor pVP2 is further processed at its carboxyl terminus to become VP2. Another ORF of segment A encodes VP5, a small (17 kDa) polypeptide reported later in 1995 by Mundt and Muller (25). Genome segment B encodes VP1, the RNA-dependant- RNA-polymerase (RdRp). In 1988, Gorbalenya & Koonin reported that VP1 of IBDV may represent the RdRp as this protein revealed the conservation of the RdRp motifs as found in single-stranded (ssRNA) + viruses (26). In 2002, Gorbalenya and others, demonstrated that VP1 coding region of IBDV contains a ubiquitous $\alpha\beta$ -palm subdomain (a core component of RdRp) comprising A, B and C sequence motifs crucial for catalysis (27).

1.5 Birnavirus replication

The cell receptor of IBDV is unknown. In chicken embryo cells, the virus multiplication cycle can vary from 10 to 36 hours (28) (29). A VP2 derived peptide-mediated virus translocation across the cytoplasmic membrane of the host cell has been suggested (30). Following host cell entry, the viral RdRp, VP1 becomes activated. A form of VP1 exists as a genomic-linked viral protein (VPg), tightly bound to the both end of the genome, which favors the viral nucleic acid replication by “strand displacement” (31). Because of the specific interaction between genome linked RdRp and IBDV genome, it has been assigned the requirement of an enzyme: putative viral transcriptase p90 (32). It was earlier accepted that a cap structure formation by enzyme (capping enzyme) activity at the early stage of transcription of Birnavirus is required for the modification of pre messenger RNA (mRNA) activities. Dobos et al., 1993 proposed that VP1 can only act as a RNA polymerase but not as a capping enzyme (33). Unlike Adenovirus or Picornavirus, Birnavirus has one protein; VP1 which functions as both virus-coded RdRp and the primer initiating RNA replication (33). Studies conducted using Baculovirus-expressed wild type IBDV, revealed the RdRp uses the template 3' end of the positive strand template to initiate the RNA synthesis by a “copy back mechanism” which ultimately falls back in to form an RNA hairpin (34). Because virus particles themselves carry all the enzymes required for replication, the transcription and replication can occur in the host cell without the need of uncoating or any form of degradation of the nucleocapsid (35). Hence the dsRNA genome is well protected inside the capsid throughout the virus cycle without being exposed to host cytoplasmic antiviral mechanisms. Such activities require that the particles be translocated across the cell membrane without disassembling during entry or undergoing only partial disassembly (36). Electron microscopy studies have shown that IBDV lacks the inner capsid that is present in all other dsRNA viruses (37). Nonetheless, the t=13 icosahedral surface of the Birnavirus particle features trimeric projection similar to those of the second layer of *Reoviridae*. As mentioned earlier, Birnavirus has been shown to become transcriptionally active in the presence of nucleotide (35), extruding non-polyadenylated mRNA through pores possibly located at the 5-fold symmetric axis in the capsid to the host cell cytoplasm (37). These viral mRNA and host ribosome then translate polyprotein and VP5 coded in genome segment A and VP1 coded in genome segment B. The polyprotein is then cleaved by the proteolytic activity of VP4 in to precursor VP2 (pVP2), VP3 and a serine protease VP4 (23). VP3 interact with the carboxyl terminal end of both pVP2 and VP1 to determine the viral morphogenesis (38)(129).

During virus maturation, the site-specific cleavage of precursor VP2 (pVP2) by VP4 enzyme activity leads to further processing of pVP2 into mature VP2 (39). VP5 is not essential for viral replication (40) but is involved in the release of the virus progeny by inducing cell lysis (41) (42).

1.6 Birnavirus structure

The virus is a single-shelled, non-enveloped virion with T=13 icosahedral capsid with a diameter around 55-60 nm (43). Three dimensional mapping has illustrated that the structure of the virus is based on a T=13 lattice and that the subunits are largely trimer clustered (44). The arrangements of the subunits give the non-spherical shape to the capsid. The outer part of the capsid has 780 subunits which are clustered in 260 trimers which protrude continuously. In the inner surface of the capsid, the trimer units appear as 200 Y-shaped features (VP3). It is likely that the outer trimers resemble the protein VP2, representing the major neutralizing epitope, and the inner trimers resemble protein VP3, which has a basic carboxyl-terminal tail likely to interact with the packed RNA and may be responsible for stabilizing functions (44). The T=13 icosahedral capsid model suggests the virion is composed of 780 copies of VP2 (50% of the virion), 600 copies of VP3 (40% of the virion), 60 copies of VP4 (6% of the virion) and VP1 constitutes 3% of virion (45).

1.7 Viral proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that IBVD contains five proteins, non-structural protein VP5 (21 Kd) and structural proteins namely VP1 (90 Kd), VP2 (40 Kd), VP3 (35 Kd) and VP4 (28 Kd) (46-48) that makes up about 3%, 51%, 40% and 6% of the viral structural protein, respectively (45).

1.7.1 Viral protein 2

The external surface of the virion is composed of trimeric subunits of the major capsid polypeptide VP2, and the inner capsid is built of dimeric subunits of VP3 (23). VP2 protein has long been identified as the foremost immunogenic component of the virus that elicits the protective neutralizing monoclonal antibodies (49). VP2 protein (441 amino acids) is the unique constituent of the icosahedral capsid (36). The sequential maturation of polypeptide VP2 to VP2 takes place only upon particle assembly (50). Expression of VP2 by itself leads to dodecahedral T=1 subviral

particles (SVP) containing 20 VP2 trimers, whereas expression of pVP2 by itself leads to irregular assemblies (51). Sequencing results confirmed that the neutralizing epitopes cluster in the variable domain which is highly hydrophobic and flanked by two major hydrophilic peaks. Three potential 'minor' antigenic sites were identified within the hydrophobic region (52). The antigenic hydrophilic regions A (amino acid 212 to 224) and B (amino acid 314 to 325) were found to constitute loops P_{BC} and P_{HI}, respectively, at the outermost part of projection domain (P) (53).

1.7.2 Viral protein 3

VP3, a dimeric in structure, is considered to be a group-specific antigen because it is recognized by monoclonal antibodies directed against VP3 from strains of both serotype 1 and 2 (54). It is a multifunctional protein involved in, i) determining the morphogenesis; acting as a scaffolding element during the assembly of the virus particle (38), interacting with the C-terminal end of the precursor VP2 and with VP1, forming a complex with VP1 leading to proper encapsidation into virions (55), ii) shielding the viral genome in stable ribonucleoprotein complexes (RNPs) which occupies the inner space of the Birnavirus virion (56), and iii) controlling the host innate antiviral responses triggered by dsRNA molecules protein kinase R (PKR) mediated apoptosis to complete the viral progeny production (57), reducing RNA slicing activity by binding both long and small RNA duplexes (58).

1.7.3 Viral protein 1

Earlier studies comparing the predicted amino acid sequence of VP1 with those of other DNA-dependent and ssRNA-dependent RNA polymerases failed to reveal any homology between VP1 and the conserved regions in these enzymes. Thus, it was proposed in 1988 by Morgan et al., that VP1 could be the viral RdRp (59). VP1 is known to exist as a VPg and as a “free” polypeptide of 90 kDa in virus particles (60, 33). Recent evidence implicating segment B in the virulence of very virulent IBDV (vvIBDV) ignited new research interest to study VP1. In spite of conflicting arguments on the molecular basis of the virulence of IBDV, some studies have showed that amino acid substitution (V4I, valine to isoleucine substitution at amino acid position 4) in VP1 can alter viral replication and pathogenicity (61). Thus, the virulence of IBDV appears to be linked to

definite sequence patterns in VP1. RdRp is an essential protein for the replication of RNA viruses that determines viral replication and tissue distribution (62). VP1 sequence of vvIBDV is phylogenetically distinct from that of all other IBDV strains (63). The recently established tri-dimensional structure of the IBDV RdRp suggested that the polypeptide chain has three major domains: i) an N-terminal domain ii) the central polymerase domain and iii) a C-terminal domain (64) (65) and all domains contribute to the viral virulence (66). VP1 is incorporated into virions through interaction with VP3 in the cytoplasm of infected cells and VP1–VP3 complex is involved in replication and packaging of the IBDV genome (67).

1.7.4 Viral protein 4

VP4 is a virus-encoded serine protease essential for processing of polyprotein into viral proteins (53, 68). For the processing of polyprotein, IBDV VP4 critical requires a serine lysine catalytic dyad (Ser652 and Lys692) (69, 70). VP4 shares properties with prokaryotic leader peptidases and other bacterial peptidases (68).

1.7.5 Viral protein 5

A second small ORF partially overlapping large ORFs polyprotein gene area has been identified. This ORF encodes VP5, a highly conserved, basic and cysteine-rich class II, non-structural, membrane protein (25). Although, VP5 protein is not present in the virion (44) and is not required for viral replication (71), it is believed to play a crucial role in cytopathogenesis involving in the release of virus progeny from the infected cell by accumulation at the host cell membrane (72) and subsequent lysis (41) (42). It also can cause cell apoptosis and/or inhibition of apoptosis at early stages of infection (73).

1.8 Pathogenesis of IBDV

To date, the host cell receptor for IBDV binding has not been identified. The oral route is considered the most common route of transmission of IBDV in chickens. Upon infection, the virus primarily replicates inside macrophages and other gut-associated lymphoid tissue. Then, the virus travels to BF via blood stream (primary viremia). The BF is the main site of viral replication occurring extensively within the first few hours of infection. The secondary and pronounced viremia occurs with secondary replication in other organs leading to disease and death (60, 74-77). In addition to B cells, IBDV also infects cells of the monocyte-macrophage lineage. In 1976,

Kaufer and Weiss provided evidence for the susceptibility of macrophages to productive infection with IBDV by showing the presence of phagocytic debris and progeny viruses in macrophages (78).

Serotype 2 strains can only replicate in bursal epithelial cells but not in bursal cells (79) (80) or in any other lymphoid cells. These strains are apathogenic for both turkeys and chickens (16). It was revealed that both pathogenic and apathogenic serotypes can infect lymphoid or primary cells but the replication of apathogenic serotypes is somehow restricted. Thus, the susceptibility of chicken lymphoid cells to IBDV does not correlate with the presence of specific binding sites (81). Another investigation indicated that IBDV host range is mainly controlled by the presence of a virus receptor composed of “N-glycosylated protein” associated with the subtle differentiation stage of B-lymphocytes represented mostly by surface immunoglobulin (Ig) M-bearing cells (77).

IBDV infection of chicken embryo fibroblasts (CEFs) leads to marked changes in membrane ion currents (K^+ outward current) which influences both the kinetics of activation and inactivation as well as Ca^{+2} dependence of activation in CEFs (82). These changes in the membrane current may cause alterations of membrane permeability, thus affecting intracellular ion homeostasis and contributing to cytolysis and death of the infected cells. IBDV replication causes apoptosis of productively infected chicken embryo cells and cells of the BF. Interestingly, it also induces apoptosis of antigen-negative cells in the vicinity of the infected cells (83). Apoptosis induced by IBDV has been identified as a multistep process involving virus replication, protein expression, and release of virions (84). VP2 is a bona fide apoptotic inducer (85) and VP5 is involved in preventing early apoptosis until virus replication is completed (73). These observations suggest that necrosis, as well as apoptosis, contributes to the rapid depletion of cells in the IBDV-infected BF.

The virus spreads rapidly through the bursal follicles and leads to severe lymphoid cell destruction in the medullary and the cortical regions and to a lesser degree, in other lymphoid organs such as cecal tonsils and spleen (86). These changes in the BF can be grossly characterized by the marked reduction of the size. The degree of inflammatory changes in the BF depends upon the type of pathogenic strain involved in infection. IBDV-mediated acute cytolytic activities in IgM-bearing B lymphocyte precursors results in reduction of circulating IgM⁺ cells (75) (87).

During the acute phase of IBDV infection, the thymus shows marked atrophy and extensive apoptosis, but recovers quickly. Yet there has been no evidence of virus replication in thymic cells (88). It was reported that T cells are resistant to IBDV infection (89). Studies examining the role of T cells in IBDV-induced immunopathogenesis and tissue recovery demonstrated that both CD4⁺ and CD8⁺ T cells infiltrate the BF reaching maximal levels at 7 days post-infection (pi) (90). These T lymphocytes control viral replication in bursal cells, however, the cytotoxicity and inflammatory cytokines can cause bursal tissue damage and delay the follicular recovery (91).

1.9 Immune responses to IBDV

1.9.1 Innate immune response

Just like many other infections, IBDV can induce an early innate immune reaction followed by an adaptive immune response. Innate immune responses are basically derived from virus activated macrophages. Macrophages are the earliest cells that encounter IBDV. In fact, gut-associated macrophages are hypothesized to take part in IBDV transportation from the digestive tract to the BF and other lymphoid tissues (78) (92) (93). During the acute phase of IBDV infection, macrophages and other immune cells, like CD4⁺ and CD8⁺, dramatically infiltrate into the site of viral replication *i.e.* BF. (90). Upregulation of pro-inflammatory cytokines (IL-1 β , TNF α , IL-6 and IL-8) gene transcription in the BF have been correlated with the presence of IBDV activated intrabursal T cells. High levels of systemic IL-6 and other pro-inflammatory cytokines and chemokines were noted during the acute phase of vvIBDV infection (94) leading to a “cytokine storm” which can potentially cause immunopathology leading to high mortality. IFN- γ produced by intrabursal T cells is a potent activator of IL-12 and in turn activate splenic macrophages to produce increasingly more pro-inflammatory cytokines and inducible nitrogen oxide (iNO) (92) (95). In addition, the macrophages that are directly infected by IBDV and the bursal macrophages that are in direct contact with viral proteins, also produce high levels of pro-inflammatory mediators (95) which promote severe bursal tissue destruction (96). Immune cell activation and cytokine production differs based on the type of IBDV strain involved in the pathogenic insult. In the acute phase of vvIBDV infection, bursal macrophages show a higher state of activation than that of classical IBDV (cIBDV) or variant IBDV (vIBDV) infection (94, 92) (97). Bursal macrophages are known to enhance the expression of mRNA encoding IL-1 β , IL-6, IL-18 cytokines and iNOS in IBDV intermediate strain infected chickens (93, 98). Compared with

vIBDV strains, cIBDV strains show higher expression of innate (IFN α and β) pro-inflammatory cytokines and immune mediators (IL-6 and iNOS) in the infected BF. Expression of mRNA coding chemokine genes, like IL-8 and MIP- α , were also higher in birds infected with classical strains (99).

1.9.2 Adaptive immune response

IBDV can induce strong adaptive immune responses against IBDV itself, while inducing a state of immunosuppression to other pathogens. Although IBDV targets immature B cells of the BF, significant clonal expansion of IBDV mature B cells has been reported following an IBDV infection (100). IBDV also activates cell-mediated immunity which is vital for virus clearance and recovery (90). The significant expression of IL-2 and IFN- γ in bursal tissue strongly suggests the activation of T cells and highlights the role of IBDV specific cytotoxic T cells in virus clearance (99).

1.10 Immunosuppressive cytokine modulation by IBDV

IBDV lytic infection of B cells leads to the destruction of antibody producing cells causing immunosuppression in birds. In addition, it was shown that splenic macrophages may play a role in reducing mitogen-induced lymphoproliferation of splenocytes from IBDV infected chickens (92). In mice, monocytes/macrophages have been identified as an important source of inhibitory cytokines, including IL-10 and TGF β . Cytokines IL-4, IL-10, IL-13 and TGF β are capable of limiting the contribution of macrophages in the inflammatory process, reducing the synthesis of pro-inflammatory cytokines such as IL-1, TNF α , IL-8, IL-6 (101, 102). Although the virus causes lytic infections in B cells, the destruction of antibody-producing cells is viewed as one of the leading reason for IBDV induced immunosuppression (76, 103) (104, 105). The role of macrophages and suppressor T cells in the immunosuppressive mechanisms have also been acknowledged. Suppressor T cells recovered from BF have shown profound inhibitory effect on *in vitro* proliferation of normal spleen cells while other splenic T cells do not inhibit the mitogenic response (106).

1.11 IBDV serotypes

IBDV has been classified into two serotypes (16). The IBDV first report from Cosgrove in 1962 (1) is considered as cIBDV and designated as serotype I. Virus neutralization (VN) tests indicated that IBDV infecting turkeys are antigenically different from chicken isolates. In 1980, McFerran et al., designated several IBDVs, isolated from chickens and turkeys, as serotype I and serotype II, respectively (17). Although serotype II viruses are non-pathogenic, they can infect both chickens and turkeys. Hence, antibodies against serotype II IBDV can be detected in many turkey and chicken flocks (107, 16). Serotype II viruses were recognized in the USA in 1982 by Jackwood and Saif (16).

1.12 IBDV strains

1.12.1 Variant strains

The RNA nature of the IBDV genome predisposes high mutation rates in virions leading to genetic drift. The high tendency of genetic drift leads to antigenically divergent strains of IBDV that escape vaccine induced immunity posing a great threat to the poultry industry worldwide (108, 109). The antigenically diverted strains which escape the immunity elicited by the classic strains are called variants. Remarkably, variant strains can establish an infection in the face of maternally derived antibodies which were previously protective against classical strains (110) (111, 112) (113). There are many reports on these variant strains breaking through maternal and active immunity barriers (114). Thus, variant strains are not cross protected by antibodies developed against conventional serotypes. Variant strains are capable of causing rapid and permanent bursal atrophy and lymphoid cell depletion. Bursal necrosis induced by variant isolates is not accompanied with an appreciable inflammatory response. It is also evident that the thymus remains unaffected by variant strains. (88). These types of infections are also classified as a sub-clinical form of IBD. Problem flocks do not have any visible clinical signs of IBD. The flock therefore may then succumb to secondary infections, exhibiting a loss in weight gain and reduced feed conversion and production performance (114). According to genotypic studies carried out in immunosuppression related problems in USA and Canada, it was revealed that the main underlying cause for these cases was sub-clinical IBDV infection with the majority being vIBDVs. (109) (114). It was also suggested that emerging variants are due to genetic drift which could be the

consequences of nucleotide mutations, strongly evidenced by nucleotide sequence analysis (109) (115).

1.12.2 Emergence of variants in the USA

In 1984, an isolate of serotype I, designated Md, was recovered from BFs with lymphoid depletions from 7 day old broilers having adequate levels of MAb to serotype I IBDV in Maryland, USA (116). It was revealed that Md virus was antigenically divergent from many vaccine and field strains existing during that period in the USA (116). A year later, in 1985, another report of antigenically distinct serotype I strains were published by Rosenberger and Cloud (117). The emergence of the antigenically variant strains escaping MAbs started causing a great challenge for the poultry industry. Subsequently, antigenic variation among isolates of IBDV, were reported from several laboratories in the USA (118-120). The economic significance of variant strains prompted advanced research on the molecular basis of the antigenic diversity of IBDV. For the identification of variant strains, reverse transcriptase polymerase chain reaction (RT-PCR) and RT-PCR- restriction enzyme (RE) analysis were introduced in addition to VN assays. Vakaria et al., 1994 compared the nucleotide sequences of the large genome segment A of four antigenically divergent IBDV strains (GLS, DS326, E/Del vs a standard vaccine strain D78) (115). The results revealed that most of the amino acid substitutions occur in the central region between residues 212 to 332, particularly in the two hydrophilic regions between residues 212 to 223 and residues 314 to 324 of VP2 protein (115). Another study reported that point mutations occur in amino acids of VP2; amino acid at positions 222 and 254 were constantly mutated in the variant viruses and importantly point mutations at amino acid position 222 defined several groups of variants emerging during the 1990s in the USA (121). Interestingly, as the poultry industry grew more variants emerged and variants in different molecular groups revealed different amino acid positions of mutations (122). Due to the highly changing nature of the virus, the requirement of frequent epidemiological surveillance arose. In 2005, Jackwood *et al.* conducted an epidemiological study to identify newly emerging viruses infecting chickens on poultry farms experiencing immunosuppression-related problems. The above study was conducted using six mutation probes of IBDV: Delaware-E (Del-E), Bursine 2, D-78, STC, G6, and T1. One or more nucleotide mutations were observed in the VP2 gene using these six mutation probes. Further, every amino acid in the hydrophilic B epitope coding region of VP2 gene, except one between 316 and 324, had

at least one substitution mutation. Phylogenetic analysis placed the majority of the viruses in previously characterized vIBDV branches as well as in newly added branches which were not previously identified (123). Of note, it was shown that a single amino acid change in the VP2 region can significantly alter the antigenicity of IBDV (124). New antigenic subtypes including new molecular clades derived from the Delaware variant lineage; DMV/4813/07, etc. were recognized as antigenically different than the Del-E strain contained in vaccines (125)

1.12.3 Canadian IBDV and emergence of variants

IBDV was first reported in New Brunswick, Canada in 1973 (126). A virus, designated as strain **Sk-1**, was isolated from pooled bursal tissue of affected 4 week-old broilers with depression and sudden death (126). The predominant histological lesion was necrosis of bursal lymphocytes. The virus was experimentally inoculated in embryonated eggs of leghorn chickens and results showed classical lesions of IBDV (126).

Vaccination of broiler breeder flocks against IBDV is employed to provide protection in neonatal broiler chicks (127). Despite this measure, excessive unexplained broiler mortalities associated with bursal lesions and bursal damage in normal birds at processing were reported in Saskatchewan by Armstrong L.D et al., 1981 (128). IBDV strain 2512 and Sk-1 were identified and isolated as the causative agent for these “problem broiler flocks” and “sub-clinical IBD infection” was successfully reproduced experimentally (128).

In the recent past, there has been a significant rise in vIBDV in the Canadian broiler chicken industry associated with production losses, clinical signs related to respiratory and enteric diseases and vaccine failures (129). In 2007, RT-PCR and VP2 gene sequencing studies revealed the presence of vIBDV in bursal samples collected from “problem broiler flocks” associated with immunosuppression related conditions across four Canadian provinces (129). The VP2 gene of different vIBDV isolates from Canada showed 95-99% homology to VP2 gene sequences of vIBDVs previously isolated from various countries; USA field isolate NC171 (98-100%), South African isolate, 05SA8 (99.2-100%), USA isolate Delaware-E related virus (98.3-100%) and USA isolate 586 (94.2% to 98.3%) (129). This study further confirmed that the majority of IBDV strains circulating in Canada were variants, and 32.8% of the total IBDV positive samples had 98-100% sequence identity to USA isolate vIBDV NC171, also called as NC171 like (NC-L) virus (129). A recent epidemiological study (conducted in Ontario in 2013) confirmed NC-L as the most prevalent

(60% in broiler chickens) vIBDV in Canada (130). Importantly, there has not been any report on vvIBDV in Canada.

1.12.4 Very virulent strains – Europe, Asia and other parts of the world

The first cases of the very virulent form of IBDV were described from Belgium in 1987 (131). Since then, the disease spread throughout other European countries (132, 133). Mortality rates as high as 100% in specific pathogen free (SPF) chickens, 60% in layers and 30% in broilers have been recorded (131) (134). In infected flocks, the clinical signs were similar to classical IBDV infection except that the disease was more pronounced and acute in individual birds and generalized in flocks. The typical lesions include hemorrhagic and enlarged or atrophied BF, degenerative liver, hemorrhages in the thigh muscles or brownish kidneys with swollen tubules (131). The European picture has been dominated for more than a decade by the emergence of vvIBDV strains. Subsequently, vvIBDV spread to many parts of the world including Europe, Asia, Africa, South America and USA (135) (136) (137) (138) (139) (140). But no reports of vvIBDV have been recorded in Canada, Australia and New Zealand (137). These studies have confirmed that vvIBDV encountered in Europe were not related to antigenic variation, but to increased virulence of the circulating IBD viral strains (141). The first published sequence of strain UK661 is now considered as the reference strain for European vvIBDV (142). Molecular, antigenic and phenotypic characteristics are regarded as the main criteria for the IBDV strain designation as the very virulent pathotype (143). The Asiatic and African vvIBDV strains show features of the common European ancestor and exhibit a high degree of genetic and antigenic homogeneity (144, 145) (146) (147). Some of the genetic determinants of pathogenicity have been illustrated. It is believed that both segment A and B of the IBDV genome influence the pathogenicity (148, 149). VP2 sequence alignments have shown that vvIBDVs share unique amino acid residues at positions 222 (Alanine), 256 (Isoleucine), 294 (Isoleucine) and 299 (Serine) (150). Highly conserved sequences have been observed in the hypervariable region of the VP2 protein of vvIBDV strains. However, it has also been suggested that VP2 is not the sole determinant of virulence (151). The ability of VP1 altering replication efficacy may play a vital role in determining virulence (148). The involvement of genome segment B that encodes the RdRp (VP1 or VPg) in virulence has been demonstrated by showing reduced pathogenicity in a rare natural segment natural re-assorted isolate with genome segment A of very virulent origin but segment B of non-virulent origin (152). Thus, it is hypothesized that sudden expansion of vvIBDV in the mid-1980s may have been initiated by the

re-assortment of its genome segment B with a mutant VP2 background (153). Assays conducted using monoclonal antibody panels revealed that vvIBDV is antigenically similar to the classical serotype I strain with only minor variations (136, 154) and classical vaccines are capable of controlling them (155, 156). An apparent re-assorting of serotype I (California vvIBDV) with an endemic serotype II virus has been reported (157). There are several examples of re-assorted vvIBDV reported all over the world (158) (159) (160), (157, 161), however in contrast to previous reports (155, 156), existing vaccines failed to completely protect against several of the re-assorted vvIBDV (159) (157).

1.12.5 vvIBDV in the USA

vvIBDV was first reported in USA from two ranches in Southern California in 2008 (136). The amino acids of VP2 recovered from the infected birds were compatible with vvIBDV isolates and the nucleotide sequencing of a fragment of the VP1 gene demonstrated the viruses have the segment B genotype associated with highly pathogenic vvIBDV. These isolates were designated rA and rB (162) (136). Despite the California incidence, vvIBDV were only found in distinct geographical areas which were unusual for the typical explosive expansion of vvIBDV in other part of the world (163). It was suggested that endemic IBDV pathotypes in the USA are potential causes of altering the severity of vvIBDV infection (164). Later, the USA had more outbreaks of IBD in California, caused by reassortant IBDV which has apparently resulted from reassorting of rA and rB vvIBDV with serotype 2 IBDV. This was the first report of a natural reassortant between IBDV of serotype 1 and 2 (157). Generally in countries where vvIBDV has been established, it rapidly spreads to highest poultry-producing regions. Since 2008 only a few cases of vvIBDV have been recognized in the USA. A viral competition study conducted using maternally immune layers challenged with a mixed infection of variant and very virulent strains, revealed that variant viruses can reduce the clinical signs and anticipated mortality after a vvIBDV infection (165).

1.12.6 South America

In South American countries; Argentina and Uruguay, main IBDV strain types are composed of typical classical, very virulent, variant and vaccine-like classical attenuated strains. Notably, the majority of the current field isolates in South America do not accurately fit into any of the previously described isolates. These strains are recognized as an independent evolutionary

lineage that have unique and conserved molecular diagnostic signature sequences in VP2 (272T, 289P, 290I, and 296F (166). These strains are denoted as distinct IBDV (167). In the case of VP1 (segment B), the highest similarity (96.4%) was found with strains that are not vvIBDV. The residue 254 S, characteristic of the antigenic variant, are rarely reported from this region (167).

1.12.7 China

IBD has been a major poultry disease in China since the first IBDV strain, CJ801 was isolated from Beijing in 1982. Currently, vvIBDV has become the most prevalent strain with major economic significance in China (144) (168). vvIBDV population sequence analysis revealed that IBDV strains prevalent in East Asia show a significant signal of positive selection and a signal of co-evolution between sites 253 and 284. Also, the change in the virulence of IBDV may result from the interaction between the virus and potential IBDV specific cell receptors. Recent Chinese very virulent isolates show several genetic variations in both segments and clustered in a distinct lineage from characteristic vvIBDs (169). Most of the Chinese field isolates of vvIBDV are antigenically similar but higher in pathogenicity than typical vvIBDV suggesting the evolution of the very virulent strain.(169).

1.13 RNA virus evolution

RNA viruses are highly mutation-prone (10^{-5} to 10^{-3} mis-incorporations per nucleotide copied) viruses with short generation times and high progeny yields (170, 171). These mis-incorporations are proofread very inefficiently or not at all (172). Also, RNA viruses generate genomic variation by homologous and non-homologous recombination and reassortment in viruses with segmented genomes (173), which will eventually permit the virus to evolve as a heterogeneous population of closely related variants characterized by one or more dominant master nucleotide genome sequence(s) called quasispecies. (174) (175). Due to the quasispecies nature, RNA viruses, acquire a significant adaptability potential through the selection of mutants best suited to a new environment. This selection allows rapid evolution of RNA viruses, which may contribute to antigenic variation and pathogenesis (171). Although, there has been many reports of quasispecies viruses (176), the evidence of quasispecies in double-stranded RNA viruses are rare. Bonneau et al. 2001 reported the presence of quasispecies in bluetongue viruses and Hsu et al., 1995 used RNA fingerprinting technique to identify quasispecies in infectious pancreatic necrosis virus, a Birnavirus related to IBDV (177) (178). IBDV quasispecies were also identified in commercial

IBDV vaccines and field isolates of IBDV, potentially contributing to the antigenic diversity (179). Moreover, homologous recombination can also contribute to the emergence of novel vvIBDV (180).

1.14 Cell culture adaptation and attenuation

Usually pathogenic bursal-derived field strains are not easily adapted to cell culture, a process which requires extensive passaging either in cell culture (181) or in the chorioallantoic membrane (CAM) or yolk sac of embryonated eggs (145). Amino acid changes occurring in the VP2 region are suggestive of attenuation during cell culture passage. Decline of the hydrophilic nature of the VP2 domain can lead to a reduction of the virulence in some very virulent strains and may allow adaptation to cell culture (145). In contrast, to above cell lines, macrophage cell lines (MQ-NCSU) are easily adaptable to cIBDV in a single passage (98). The mechanism of this altered tropism is unknown. It is believed that the virus might have changed amino acids in the protein other than VP2, and continue receptor binding and post-attachment viral entry processes (98).

1.15 Diagnosis of IBD

Clinical signs and gross lesions can be used as preliminary tools of diagnosis. But in a situation of a sub-clinical immunosuppression which goes unnoticed, confirmatory diagnosis is accomplished by isolation of the live virus or detection of viral antigen in tissue. Virus isolation is done in the CAM in embryonated chicken eggs which is considered as the most sensitive route (182). Many IBDV isolates can be adapted to primary or continuous cell lines of chicken origin. (183). Once the virus is isolated, it may be recognized using known antiserum of IBDV by many methods namely, VN, fluorescence antibody technique (FAT), antigen captured enzyme linked immunosorbent assay (ELISA) and agar gel precipitation test (AGPT).

In tissue, the virus can be detected by antigen captured ELISA (184) (119), AGPT, FAT, nucleic acid probes(185) and RT-PCR and its derivatives. The early diagnosis of different antigenic strains was demonstrated by VN tests. (118) (186) (119). VN test is also employed in many cross-protection studies in IBDV, and is done for determination of immunogenicity of different virus strains (110, 112). The RT-PCR has become a recent trend of diagnosis of IBDV in many studies. (114) (187) (188) (189, 190). The primary focus of the RT-PCR is on the variable sequence region of the VP2 protein, which is known to encode one or more neutralizing epitopes of the virus. This

can also be used for detection and identification of different strains of IBDV (191) (192) (193) along with identification of nucleotide similarities or diversity of the viruses. Real-time PCR is also used as a modern molecular technique for IBDV diagnosis which enables both detection and quantification of the DNA sample. (194). Moreover, RT-PCR restriction endonuclease (RE) and RT-PCR restriction fragment length polymorphism (RELP) are used for PCR-based molecular detection and differentiation of IBDV in modern laboratories (114, 195) (196). DNA sequencing of RT-PCR products and genome segments are significant since it facilitates calculation of pairwise sequence identities and construction of phylogenetic trees (197) (114). There are many serological techniques that can be carried out to for the detection of IBDV, namely ELISA, AGPT and serum neutralization (198).

1.16 Control of IBDV

1.16.1 Vaccine

Bloom et al., 1989 (3) classified methods of vaccine development from the naturally infectious agent into three primary areas: i) inactivated pathogen/killed, ii) native or altered antigenic subunits of the pathogens and iii) developing live attenuated strains of the pathogens. Recent research in vaccine development has focus on genetically engineered vaccines to target the protective epitopes, recombination with vectors or artificially synthesized viral proteins (199) (200).

1.16.2 Live attenuated and killed vaccines

In 1968, the first vaccines against IBDV were developed based on a live attenuated mild strain of IBDV. Bursa Vac (201) and IBD Blen (202) were the first commercially available vaccines, which prevented clinical signs of the disease but still caused bursal damage. Live attenuated virus vaccines mimic the infection to induce host immunity for reducing the clinical disease or immunosuppression. In general, virus vaccines are attenuated by serial passage in cell culture and embryonated eggs (203). In 1967, Moulthrop and Carol were able to adapt a mild isolate of IBDV to the chicken embryo-system and it ultimately became the first licensed vaccine, “Bursa-Vac” (204). Classical virulent strains of IBDV are used as vaccine candidates. Highly attenuated vaccines are called “mild” vaccines which exhibit a low level of immunogenicity due to great alteration of the parent virus. These are less efficient against vvIBDV and easily neutralized by

MAB. Intermediate, intermediate plus and hot vaccines show improved antigenicity and have better efficiency against MAB and vvIBDV. However, there is a risk of conversion of the less attenuated hot vaccines back to a virulent strain and other side effects such as vaccines induced bursal damage or immunosuppression (205, 156). Although killed vaccines have no risk of bursal damage and immunosuppression, they are less efficient in immunogenicity unless combined with a supporting adjuvant. Vaccination with killed vaccines before the start of egg lay provides passive immunity to the progeny by means of MABs (reviewed in Muller H et al., 2012). The discovery of maternal transfer of IBDV antibodies from the dam to the progeny opened the doors to the concept of **“hyper-immunization of broiler breeders”** on which the current vaccination programs are based (8, 9). In fact, broiler breeders are vaccinated with a series of live, intermediate and killed IBDV vaccines. This is done to maximize the number of antibodies which progeny receive via the yolk sac. Since the level of MAB circulating in the progeny determines the amount of protection from early exposure to environmental pathogenic IBDVs (206, 207). Live attenuated vaccines are widely used in breeder immunization programs in Canada as prophylaxis of IBD, followed by the killed vaccines at a later age. After hatching, some producers immunize broiler chicks with live attenuated vaccines. The time-point of vaccination is crucial as persisting MABs might neutralize the vaccine. The titers may vary considerably within a flock and revaccinations may be necessary (208).

1.16.3 Genetically engineered vaccines

With advanced knowledge of the IBDV genome, genetically engineered vaccines have widely experimented with the aim of generating attenuated IBDV potentially appreciable as a vaccine. “Attenuated mutant IBDV” was generated from vvIBDV by site-directed mutagenesis of nucleotide sequences encoding specific amino acids in IBDV structural protein VP2 (199) (209). Nevertheless, the reversion of these mutants to virulence has also been experienced during some trials (199). “Chimeric viruses”, in which the genomic region of virulent serotype I IBDV is replaced by the corresponding genomic area of serotype II (apathogenic), were shown to cause mild depletion of bursal cells in susceptible chickens (210). Moreover, experimental re-assortment of serotypes I and II was performed to produce ‘inter-serotypes reassortant’ IBDV vaccines (211). These vaccines were able to induce high titers of neutralizing antibodies while causing less damage to the BF. In spite of extensive investigation on IBD vaccines, none of these genetically engineered products have yet reached the market.

1.16.4 Subunit vaccines

VP2 is the major protective viral antigen where neutralizing epitopes are conformationally dependent. Recombinant VP2 (rVP2) is used in subunit vaccine production. Baculovirus-expressed IBDV derived assemblies as VP2 capsids, VPX (also called pVP2) tubules and polyprotein (PP) were tested as sub-unit vaccines. Among them, the immunogenicity and protective capability were higher in the order of; VP2 assemblies of icosahedral capsids (virus-like particles), PP and VPX tubules in SPF chickens (200). In experimental vaccination studies “fusion protein consisting of VP2 and IL-2” have been reported as an enhanced product of immunogenicity (212).

1.16.5 Immune complex vaccines

In 1995, C.E. Whitfill introduced the concept of an “immune complex” IBD vaccine (Icx-IBD), constructed by mixing IBDV antibodies with IBDV (213). Immune complexes are antigen-antibody complexes which are formed when an antigen encounters its specific antibody (214). When a bird is vaccinated with an Icx-IBD vaccine, some proportion of it is trapped in B follicular dendritic cells via binding to Fc receptors³ and complement C3 receptors (215). Vaccine antigens are preserved this way is suggested to play a crucial role in inducing a prolong immunity and sufficient levels of B cell memory (216). It is hypothesized that specific IBDV antibodies bound to vaccine IBDV antigen protect the IBDV antigen from being neutralized by host MAb. The delayed release of the Icx-IBD from follicular dendritic cells is also helpful to avoid very high levels of host MAb, which is highly crucial for protecting the vaccine viral load to induce immunity (216).

1.16.6 Live vector vaccines

The invention of recombinant vector vaccines was a remarkable accomplishment in genetically engineered vaccine production. It is produced by inserting specific gene sequences of one organism; donor, to a genome of another organism; recipient. Vector vaccines are supposed to elicit protective immunity against both organisms. Herpes virus of turkey (HVT), one of the most popular viral vaccine candidates in poultry, has been widely used in conventional vaccination against Marek's disease since the early 1970s (217). Because of the lack of pathogenicity, availability as cell-free and cell-associated preparations, HVT was recognized as a potential vector carrying many avian pathogens. The concept of recombinant HVT (rHVT), was originally revealed in the USA (218) (219). rHVT vaccines are safe and not affected by the presence of MAb. rHVT vector vaccines are known to induce both humoral and cell-mediated immunity with a long lasting protection (220). In 1995, the first report which describes the induction of full protection against IBD with a single inoculation of a rHVT-IBD vaccine was published in France (221). Since then, *in-ovo* vaccination of rHVT-IBD has been adopted as an efficient method of choice for controlling IBDV infections in many hatcheries (222) (223) (220) (224). Also, fowlpox virus (225), Newcastle disease virus (226), Marek's disease virus (227), avian adenovirus (228) and T4 bacteriophage (229) have been used as vector viruses for expressing the VP2 protein of IBDV.

1.16.7 Vaccine challenges

Rapid changing antigenicity and virulence of the virus are the most challenging factors in controlling IBDV by vaccination (163, 208, 230, 52, 143). The rise of antigenic variants has complicated the control of IBDV by vaccination since the early 1980s (114). The USA and Canada are the major countries involved with the struggle of exploring new vaccine solutions to control antigenic variants inducing damage in the BF of chickens, even in chicks from well-vaccinated hens (129) (109). Variant strains do not cause overt clinical disease but induce severe immunosuppression. The degree of immunosuppression varies depending on the virulence of the virus strain and when the infection occurs. Immunosuppression is greater when infection occurs close to the time of hatch and because the birds are a young age, the immunosuppression is permanent. The immunosuppression resulting from an IBDV infection is the underlying cause of many cases of the respiratory and enteric disease in chickens and vaccination failures (129) (109).

1.17 Biosecurity measures

Given that IBDV is a non-enveloped virus, it is known to be resistant to many physical and chemical agents. In fact, it is resistant to heat (less than 60°C), chloroform, ether, extreme P^H (3-12) and some phenol components (7). Thus, the virus exists for a long time in poultry barns (231) and the contaminated environment increases the incidence of disease (232). In addition, the virus has a higher tendency to recur on the same premises in successive flocks of broiler chickens (233). Intranasal, intraocular, and oral routes are all effective in establishing infection in a contaminated environment (234) (7). Only chlorine and aldehyde containing disinfectants are effective against IBDV (235). The infectivity of the virus is markedly reduced by formaldehyde (13) acting on proteins by denaturation and on nucleic acids by alkylation (Maris P et al., 1995 OIE article). However, use of chlorine and aldehyde have food safety issues and management difficulties. New studies are in progress for identifying new disinfectants that are able to penetrate chicken litter and inactivate enveloped as well as non-enveloped viruses (236). Although vaccination is the best option for IBD control, minimization of virus exposure should be controlled by proper management practices. Complete barn clean-out of organic matter, new litter for each subsequent flock, control of traffic (people, equipment, vehicles, etc.) onto the farm and most importantly a comprehensive biosecurity program.

1.18 Objectives

1. The first objective was to study the efficacy of broiler breeder vaccine induced maternal antibodies against vIBDV-SK09 in Canadian broiler chicken industry.
2. The second objective was to study the immunosuppressive effects of vIBDV SK09 in commercial broilers.
3. The third objective was to study the efficacy of commercially available broiler vaccines against vIBDV-SK09 infection.
4. The fourth objective was to investigate the potential of using vIBDV SK09 as a vaccine candidate for controlling vIBDVs in the Canadian broiler chicken industry.

CHAPTER 2 : EFFICACY OF BROILER BREEDER VACCINE INDUCED MATERNAL ANTIBODIES AGAINST VARIANT INFECTIOUS BURSAL DISEASE VIRUS (VIBDV) SK09 IN THE CANADIAN BROILER CHICKEN INDUSTRY

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2.1 Abstract

Recent studies have demonstrated that the majority of IBDV strains circulating in Canada are “variants”. The aim of this study was to characterize the immunosuppressive effects of vIBDV-SK09, the most prevalent vIBDV in Canada, in SPF leghorns, MAb free broilers and commercially available maternally immune broilers. In experiments 1 and 2, SPF leghorns and maternally immune broilers were challenged with vIBDV-SK09 at day 6 or days 6 and 9 or days 6, 9 or 12 of age showed significant bursal atrophy with severe lymphoid lesions. Although SPF leghorns showed early lymphoid destruction when compared to maternally immune broilers, the severity of ultimate bursal damage by 35 days of age had no significant differences between groups. It was also revealed a similar severity in bursal atrophy, irrespective of the frequency of IBDV challenges received. In experiments 3 and 4, MAb free and maternally immune broilers were challenged at 1 day of age. MAb free broilers, as expected, showed severe bursal atrophy earlier than maternally immune broilers. However it is noteworthy that irrespective of the higher MAb levels at hatch, vIBDV-SK09 challenged, maternally immune broilers had severe bursal atrophy and significant histopathological lesions of lymphoid depletion by 19 days of age and lesions have progressed into more severe form by 35 days of age. The data suggests that the most abundant Canadian vIBDV-SK09 is capable of breaking through MAb produced by broiler breeder parent vaccinations causing severe lymphoid depletion in the BF which may explain growing field reports of immunosuppression related broiler cases isolated with vIBDV strains.

2.2 Introduction

IBD is a viral disease with a considerable economic impact on the poultry industry. IBDV causes immunosuppression in young chickens by infecting and destroying immature B lymphocytes in the BF (76). The disease has also been designated as Gumboro disease because the etiological agent was first isolated in broiler chickens in Gumboro, Delaware, USA in 1962 (201); while in Canada, the disease was first reported in 1973 (126). The discovery of maternal transfer of IBDV antibodies from the dam to the progeny opened the doors to the concept of “hyper-immunization of broiler breeders” on which the current vaccination programs are greatly emphasized (67, 68). In this method, breeders are vaccinated with series of live, intermediate and killed IBDV vaccines. This is particularly practiced to maximize the quantity of antibodies which the progeny receive via the yolk sac since the level of MAb circulating in chicks determines the

protection them self from the early exposure to environmental pathogenic IBDVs (46, 200). Live attenuated vaccines are largely used in breeder immunization programs in Canada as the prophylaxis of IBD, followed by killed vaccines at a later age.

Typically, whenever IBDV becomes established geographically, it spreads rapidly and efforts at eradication have so far been unsuccessful. The stability of the virus is mainly determined by high resistance to many physical and chemical agent which induce prolonged survivability in the environment (237) and the high tendency of reoccurrence of the virus in successive broiler chicken flocks is significantly higher (233). The survivability of the virus is also maintained at a greater rate due to the presence of the different antigenic and pathogenic subtypes of the virus that can escape the immunity induced by conventional vaccines. Re-assortment of serotype I and II can result in new forms of very virulent strains (157) whereas recombination and point mutations are considered as leading causes of antigenic variations of IBDV. The main structural capsid polypeptide, VP2, is the foremost immunogenic component of the virus and elicits protective neutralizing antibodies (113). The hypervariable region of the VP2 frequently mutates allowing antigenic shifts of the virus (238). Subsequently, the antigenically varied viruses support the process of evolution and successfully go through the natural selection. Thus, a standardized vaccination protocol against IBDV is not ideal for all situations. Since the vIBDV acquire antigenically unique features, they can escape the maternal antibodies induced by classical vaccine strains. vIBDV infection is typically subclinical resulting in rapid and permanent bursal atrophy with severe lymphoid cell depletion without a significant inflammatory response. The thymus remains unaffected by the vIBDV infection (88). The subclinical form of IBD does not produce visible clinical signs of classic disease, however; flocks show reduced weight gain, poor feed conversion and succumb to secondary infections (129). Recent studies have revealed that vIBDV continues to pose a threat to the poultry industry particularly in the USA and Canada. In 2005, a molecular and epidemiological study conducted across the major poultry producing states of the USA identified a number of previously characterized vIBDV and new vIBDV with new nucleotide sequences of VP2 that had not been reported previously (109). Another study was conducted investigating immunosuppression related cases in broilers across four Canadian provinces. It was revealed that the majority of the IBDV strains circulating in the country were vIBDVs (129). The VP2 genes of different variants isolated from Canada showed 95-99% homology to VP2 gene sequences of vIBDV isolates from the USA (129). Another study conducted in Ontario in 2013

also confirmed the presence of vIBDV s that are closely related to some of the USA variants (130). However, to date, there is no work on characterizing virulence of vIBDV isolated in the poultry industry in Canada. The objective of this study was to characterize the most prevalent vIBDV-SK09 circulating in Canada (98.3% nucleotide sequence identity with the USA isolate NC171) in SPF leghorns, MAb free broilers and maternally immune commercial broilers. Three types of chickens selected in this study represented variabilities in genetic susceptibility to vIBDV and the effectiveness of MAb against vIBDV. In addition, experiments described here demonstrated the effect of single vs multiple exposure of the most prevalent vIBDV-SK09 in neonatal broiler chickens in the Canadian industry.

2.3 Materials and Methods

2.3.1 Preparation of vIBDV-SK09 for animal experiments

Canadian vIBDV-SK09 strain was used in all of the experimental studies. The BF collected from commercial broilers with suspected IBD was tested with PCR and VP2 gene sequencing (mentioned below) to confirm the presence of vIBDV-SK09. The clinical isolates were then passaged in 17-day-old SPF leghorns for 3 days. The birds were orally infected, reared in a biosafety level 2 facility and BF were collected at 3 days post infection (pi). The BF were pooled and homogenates were prepared. Briefly, BF were cut into 2-4 mm pieces using a sterile scalpel blade and suspended in 40% (w/v) Dulbecco's Modified Eagle medium: nutrient mixture F-12 (DMEM/F-12) (Life Technologies, Burlington, ON) supplemented with 0.05 % (w/v) gentamicin.

2.3.2 Conventional PCR and VP2 genome sequencing of the virus inoculum

RT-PCR and sequencing of the VP2 hypervariable region were conducted for vIBDV-SK09 isolate as previously described (129). RNA was extracted from the bursal homogenate using the RNeasy kit (Qiagen, Mississauga, ON) as per the manufacturer's instructions. The RT-PCR was carried out in 50 µl reactions using One-Step RT-PCR Kit (Qiagen, Mississauga, ON). The nucleotide sequences of the fragments were confirmed by DNA sequencing (Macrogen Seoul, South Korea). The nucleotide sequences were further analyzed in the chromatogram to confirm the purity of the inoculums.

2.3.3 Titration of the virus inoculum

Bursal homogenate was titrated in SPF embryos as described previously (136). Briefly, 10-day-old embryonated SPF chicken eggs were inoculated with 0.1 ml of filtered (45 nm pore size) bursal homogenate diluted 10-fold in pH 7 phosphate buffered saline (PBS) , containing 10 µg/ml gentamicin sulfate (Gibco, Invitrogen, Carlsbad, CA) via the CAM. The embryo infective dose 50 (EID₅₀) was determined using Reed and Munch method (136). The VP2 sequence of the IBDV isolate was conducted to confirm identity.

2.3.4 ELISA

Sera samples were tested for antibodies against IBDV using a commercial ELISA kit according to manufacturer's instructions (IDEXX, Westbrook, ME).

2.3.5 Bursal weight to body weight percentage (BBW) and bursal histopathology

BBW of each bird was determined at necropsy and calculated as bursal weight (g) /body weight (g) x 100. Sections of BF were processed for histopathology by fixing in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin and sectioned at 5 µm and stained with hematoxylin and eosin (H&E). Sections of BF were graded on the basis of lymphoid atrophy as follows; 0 = no visible lesions; 1 = mild, focal to multifocal lymphoid atrophy; 2 = moderate, multifocal lymphoid atrophy; and 3 = severe, diffuse lymphoid atrophy (Figure 2-1).

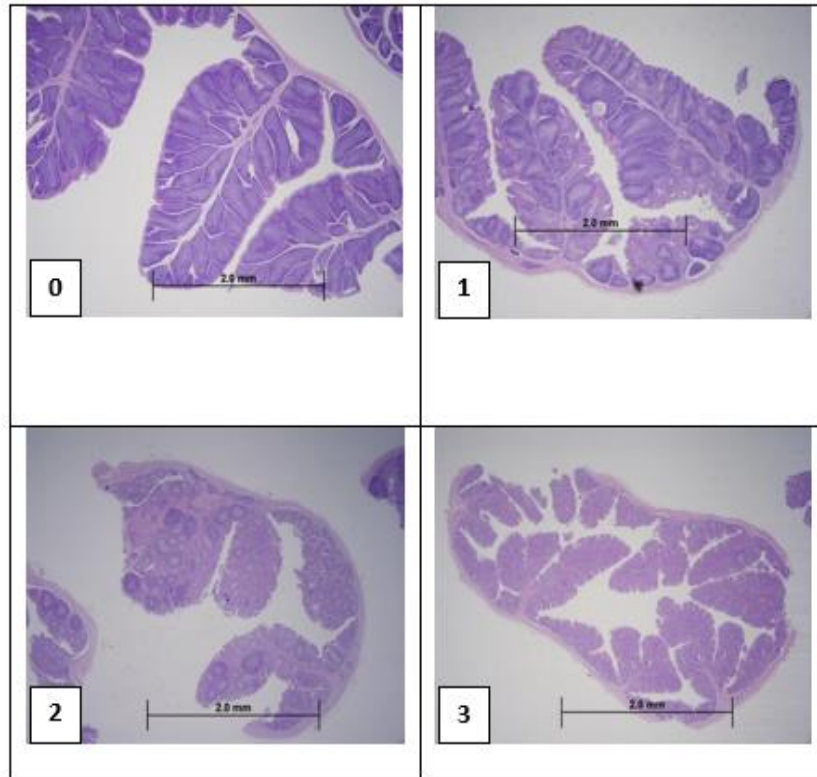


Figure 2-1: Histopathological scoring of BF following exposure of birds to vIBDV BF was graded on the basis of lymphoid atrophy as follows; 0 = no visible lesions; 1 = mild, focal to multifocal lymphoid atrophy; 2 = moderate, multifocal lymphoid atrophy; and 3 = severe, diffuse lymphoid atrophy.

2.3.6 Maintenance of broilers, broiler breeders, and SPF leghorn chickens

This work was approved by the University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan following the guidelines of Canadian Council on Animal. Day-old broiler chicks (Ross 308) were obtained from commercial hatcheries (Prairie Pride Chick Sales Ltd. and Grandora and Lilydale, Inc. Wynyard, SK). Groups of day-old chicks were randomly allocated to animal isolation rooms at the Animal Care Unit (ACU), Western College of Veterinary Medicine, University of Saskatchewan. Water and commercial broiler starter ration (Co-Op Feeds, Saskatoon, SK) containing 0.0125% Amprolium was provided *ad libitum*. Broilers were raised at 32-35 °C for the first week of life; thereafter the temperature was decreased 0.5 °C per d until a room temperature of 21 °C was reached. The light was provided for 24 h per

d during days 0 to 2 post-hatch. Darkness was introduced at day 3 post-hatch with 1 h of dark added daily until 7-8 h of darkness was achieved. This was maintained for the duration of the trial. Each room was ventilated with filtered, non-recirculated air at a rate of 15-20 changes per h. Air pressure differentials (*i.e.* negative pressure) and strict sanitation were maintained in a level 2 isolation facility. The commercial broiler breeder parent flocks of these broiler chickens had been vaccinated against IBD at 14 d of age (S-706 Bursal, Merial, Gainesville, GA), 21 d of age (Univax Plus, Merck Animal Health, Summit, NJ), 8 weeks of age (Bursa Blen M, Merial, Gainesville, GA), 10 weeks of age (Maximum 6, Ceva Biomune, Lenexa, KS) and at 18 weeks (Avi-Pro 432 ND-IB2-BD3 REO, Lohmann Animal Health International, Winslow, ME).

Naïve broiler breeder parents, used for breeding stock of MAb free broilers, were received from Aviagen, Inc. (Huntsville, AL). At hatch, all birds were vaccinated against Marek's Disease (Intervet Rismavac, Summit, NJ; Select HVT, West Perth, WA). Females received infrared break treatments while males received both toe trimming and infrared beak treatments. Birds were housed at the ACU and feeding and lighting programs were conducted according to the Aviagen, Inc. guidelines. Briefly, chick starter plus 0.0125% Amprolium (Co-Op Feeds, Saskatoon, SK) was feed *ad libitum* until 7 d of age thereafter feed was restricted. Birds were then fed with a grower ration (Co-Op Feeds, Saskatoon, SK) until 22 weeks of age and then a breeder ration (Co-Op Feeds, Saskatoon, SK). Birds were individually weighed weekly to determine the amounts to feed per bird necessary to reach the target body. Males were reared separately from females until 16 weeks of age. Males were housed and fed in individual cages, with one male released among the females for mating. Males were rotated from cages to the floor after 2 d of mating. Light intensity was maintained at 10-20 lux until 21 weeks of age and then increased to 30-60 lux. Initially, 24 h of light was provided, which was then decreased until 8 h of light was obtained at 10 d of age. Light stimulation began at 21 weeks of age with 11 h of light provided initially, then increased until 13 h of light was provided at 25 weeks of age to induce egg production. Serum samples were collected at 13, 16, 25, 35 and 45 weeks of age for IBDV, and chicken anemia virus antibody testing.

SPF eggs were obtained from Sunrise Farms, Inc. (Catskill, NY) and incubated in ACU facilities. Water and commercial broiler starter ration (Co-Op Feeds, Saskatoon, Saskatchewan) containing 0.0125% Amprolium was provided *ad libitum*.

2.3.7 Experimental Design

a) Experiment 1: Detrimental effects of single vs repeated exposure of vIBDV-SK09 SPF leghorns

The aim of this experiment was to study the effect of vIBDV-SK09 virus in SPF leghorns, with high genetic susceptibility and immunologically susceptible as they are free of MAb of IBDV. The trials were also designed to compare the effect of single vs multiple exposures of the virus by which we can evaluate the different time points of exposure at the field conditions. Day-old chicks were divided into 4 groups of 15 (Table 2-1-A). An additional 15 birds were bled to test the antibodies for IBDV. The groups received saline or vIBDV-SK09 strain (10^1 EID₅₀/bird) virus orally at different ages as follows; Group 1 – control, saline at 6 d of age, Group 2 – single challenge at 6 d of age, Group 3 - two challenges at 6 d and 9 d of age and Group 4 - three challenges at 6 d, 9 d and 12 d of age. The birds were sacrificed for sampling, sera, BW and histology, (n=2 to 5) at 9, 12, 19 and 35 d of age. IBDV antibody ELISA, BW and histopathological scorings of BF were conducted as mentioned above. BF collected at 19 d of age was used to re-confirm the presence of challenged vIBDV-SK09 strain.

b) Experiment 2: Detrimental effects of single vs repeated exposure to vIBDV-SK09 on maternally immune broilers from IBD vaccinated broiler breeder parents

This is a more comprehensive study to demonstrate the effect of vIBDV-SK09 strain in broilers which can be extrapolated into commercial broilers in the field. Importantly, it was targeted to see the protective ability of MAb against the Canadian vIBDV-SK09 strain. All experimental procedures, numbers of birds and challenge doses are similar to experiment 1 (Table 2-1-A).

c) Experiment 3: Detrimental effects of single exposure of vIBDV-SK09 on maternally non-immune broilers derived from IBD non-vaccinated broiler breeder parents

This experiment was designed to demonstrate the detrimental effects of vIBDV-SK09 strain in broilers who are immunologically susceptible since they are derived from parents not vaccinated against IBD (hence no MAb against IBDV at hatch). It was also aimed to compare the results of experiment 2 and to evaluate the effect of MAb in broilers with a similar genetic background. In

contrast to experiments 1 and 2, here we exposed the birds to the virus as early as 1 d of age. Twenty chicks were euthanized immediately after hatch for IBDV antibody testing. The birds were divided into two groups of 60 birds each (Table 2-1-B). Group 1 (control group) received saline orally at 1 d of age whereas Group 2 received 3×10^3 EID₅₀ of vIBDV-SK09 strain similarly. Twenty birds were sacrificed at each time point; 7, 19, and 35 d of age. Sampling, data processing, and challenge virus re-confirmation were done as mentioned in experiments 1 and 2.

d) Experiment 4: Detrimental effects of single exposure of IBDV variant strain SK09 in maternally immune broilers derived from IBD vaccinated broiler breeder parents

This is the most generalizable form of experiment 3. Broiler chicks from broiler breeder parents vaccinated with IBD were used in this experiment. The broiler chicks were challenged with vIBDV-SK09 strain at 1 d of age; a time where the birds comprise of very high levels of MAb. Thus, the main objective was to observe the detrimental effects of a single challenge of vIBDV-SK09 strain at 1 d of age where a maximum level of passive protection against IBDV should be present. The remainder of the procedures were conducted as that in experiment 3 (Table 2-1-B).

Table 2-1: Detrimental effects of single or multiple exposure of vIBDV-SK09 in SPF leghorns, MAb free and maternally immune broilers

A		
Group (n=15)	vIBDV-SK09 inoculation age (d)	Sample collection age (d)
1	N/A	9, 12, 19, 35
2	6	9, 12, 19, 35
3	6, 9	12, 19, 35
4	6, 9, 12	19, 35
B		
Group (n=60)	vIBDV-SK09 inoculation age (d)	Sample collection age (d) n=20/time point
1	N/A	7, 19, 35
2	1	7, 19, 35

2.3.8 Statistical analysis

The BBW, histopathological score, and antibody titer against IBDV were analyzed using Wilcoxon Rank Sum Test and Kruskal–Wallis with Dunn’s post hoc. Two-tailed tests conducted with 95% confidence intervals. PRISM-Graph pad 5 was used for graphical presentations.

2. 4 Results

a) Experiment 1: Detrimental effects of single vs repeated exposure of IBDV variant strain SK09 in SPF leghorns

The control group was negative for IBDV antibodies throughout the trial. All virus challenged SPF birds showed a significant increase of IBDV antibody titers following exposure. The level of seroconversion was correlated with the number of virus challenges the birds received. The geometric mean (GM) \pm standard deviation (SD) antibody titers at 19 d of age were 1034 ± 3146 , 1923 ± 2873 and 7862 ± 7869 whereas at 35 d of age were 2896 ± 1340 , 8323 ± 11769 and 18807 ± 1279 in the groups of single, double and triple challenged groups respectively (Figure 2-2). The control group had significantly higher BBW and lower histological lesions counts on 12, 19 and 35 d of age compared with the challenged groups ($P < 0.05$) (Figures 2-3 & 2-4). All three

challenged groups showed lymphoid atrophy in a similar level irrespective of the number of challenges received.

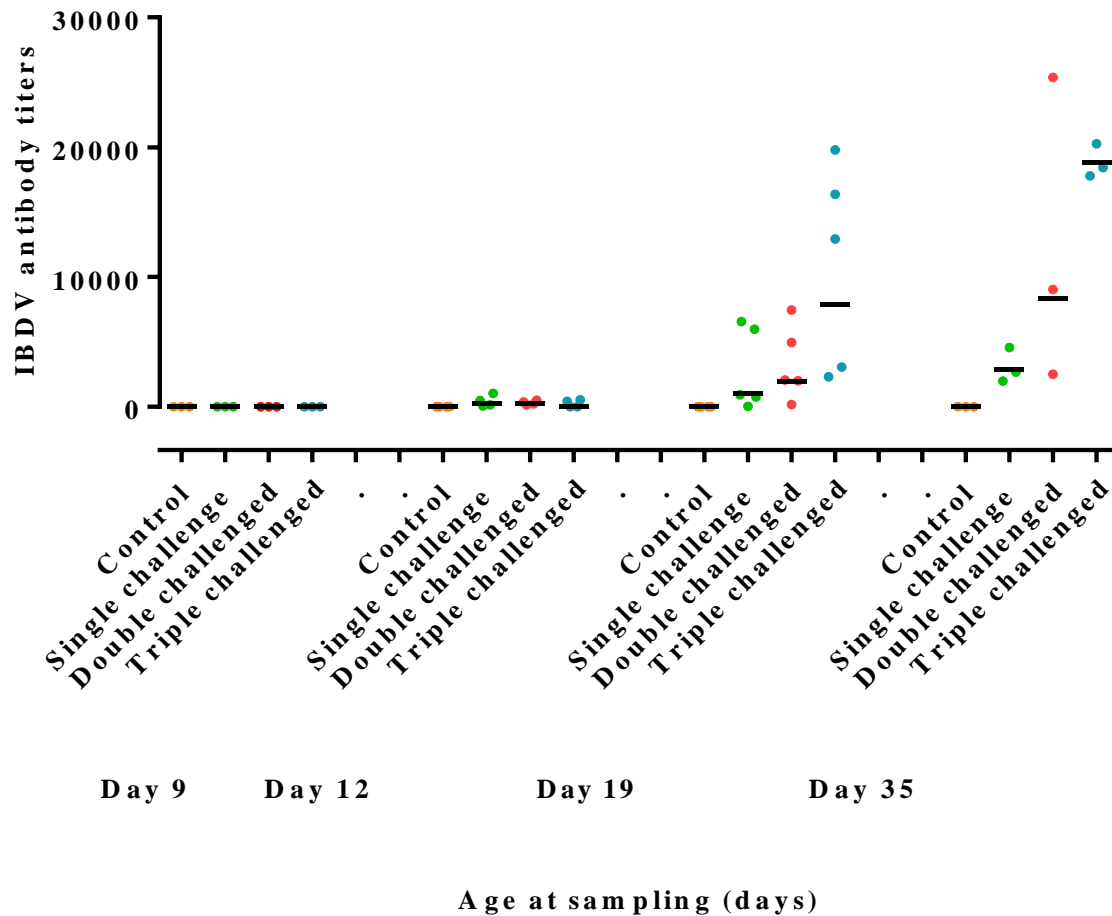


Figure 2-2: Experiment 1; Serum antibody titers to IBDV of the control group and challenged groups. Each data point represents individual Ab titers and horizontal bar indicates geometric mean titers.

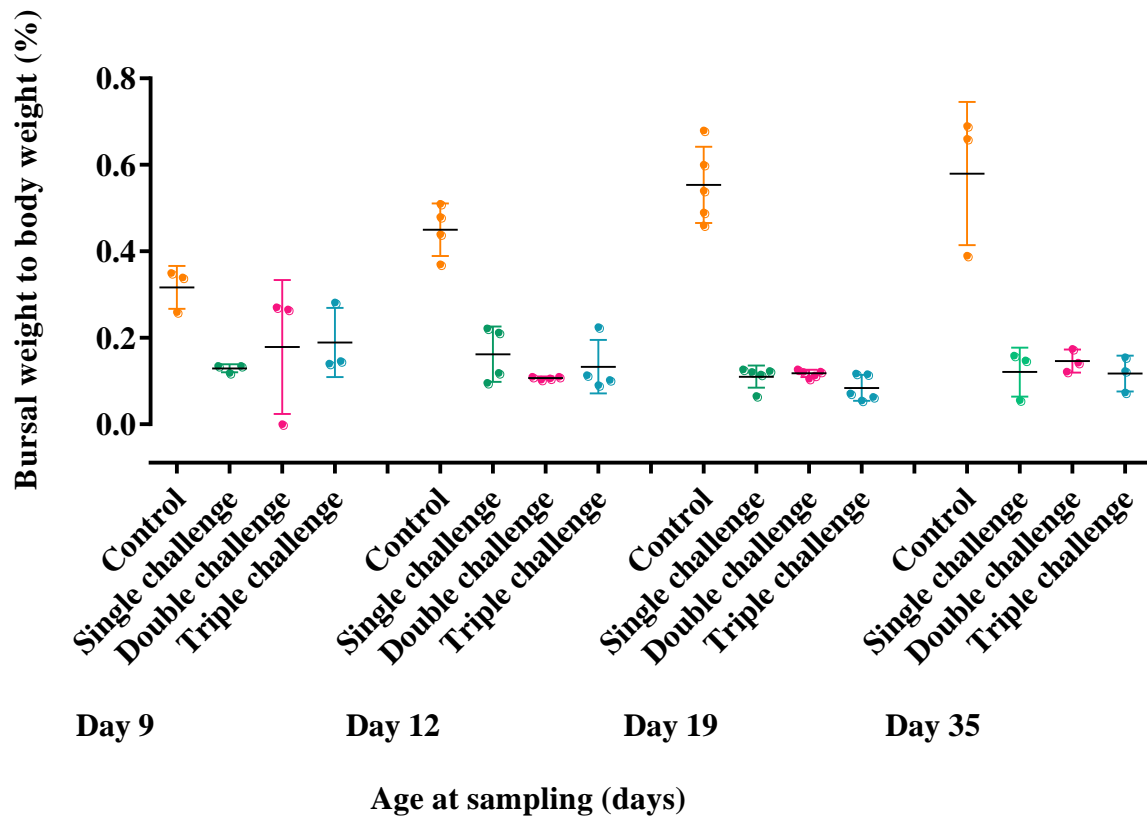


Figure 2-3: Experiment 1 - Bursal weight to body weight percentage (BBW) of SPF leghorns at 12, 19 and 35 d of age. At each age point, 3-5 birds were euthanized from the control group and challenged groups and BBWs were measured. Each data point = individual BBW data, Horizontal bar= mean, vertical lines= SD.

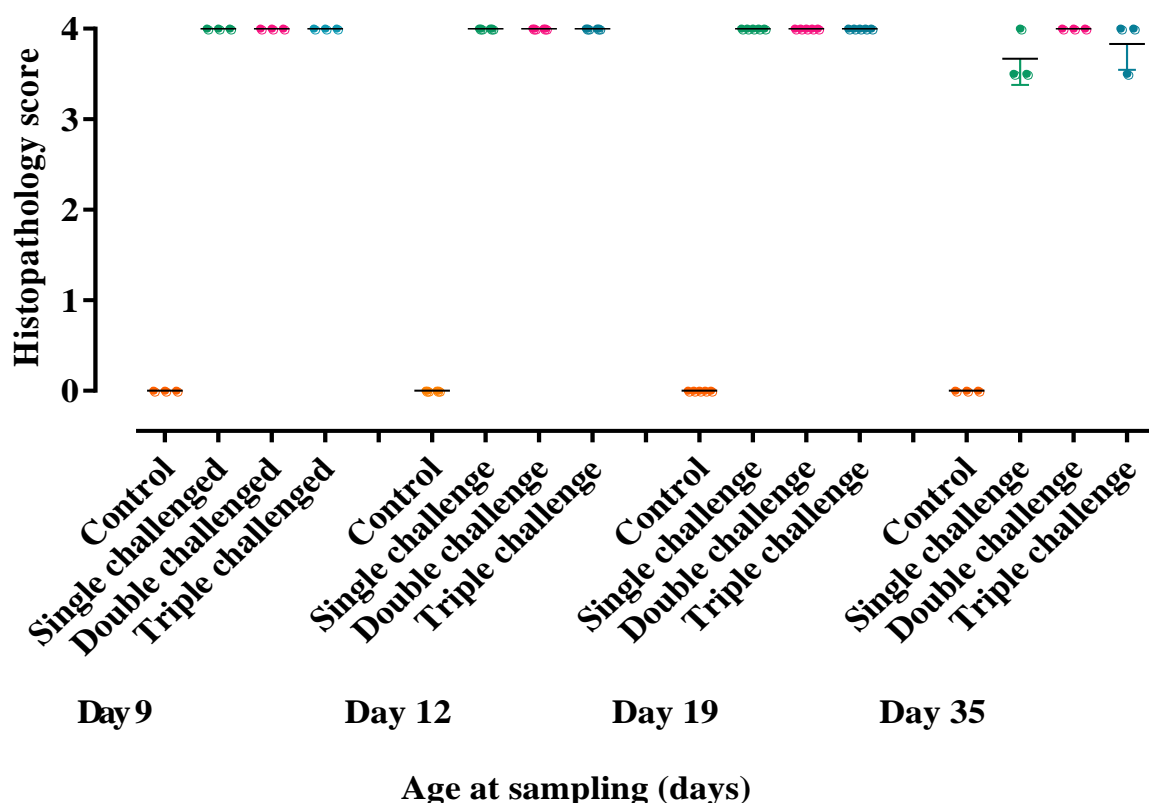


Figure 2-4: Experiment 1; Histopathological scores of BF of SPF leghorns at 9, 12, 19 and 35 d of age. At each age, 3-5 birds were euthanized from the control group and challenged groups and BF samples were taken. The BF were fixed in formalin and histopathological analysis was conducted. The scoring was conducted according to Figure 1. Each data point = individual histopathological score, Horizontal bar = mean, vertical lines = SD.

b) Experiment 2: Detrimental effects of single vs repeated exposure of vIBDV-SK09 in maternally immune broilers from IBD vaccinated broiler breeder parents

The GM (\pm SD) titer of MAb against IBDV was $9\,708 \pm 3\,460$ at the time of hatch. In the control group, antibodies against IBDV declined to 182 ± 134 and to 10 ± 46 by 19 and 35 d of age, respectively. Group 2, challenged at 6 d of age, had a GM titer of 24.6 ± 110 at 19 d of age. However, Group 2 showed a significant increase in antibody titers; $4\,348 \pm 888$ by 35 d of age than that of the control group ($P < 0.05$). Interestingly, there were no significant differences in GM antibody titers among the groups of multiple challenges ($P < 0.05$) (Figure 2-5). At day 35, mean

BBWs of the control group was (0.19) significantly higher than the mean BBW of single (0.05), two (0.05) and three (0.04) times challenged groups (0.05). There was no difference between the BBWs among the groups irrespective of the number of challenges (Figure 2-6). Histopathological observation revealed a similar pattern where control group had normal lymphoid tissue architecture whereas challenged groups showed severe lymphoid tissue depletion by 35 d of age (Figure 2-7).

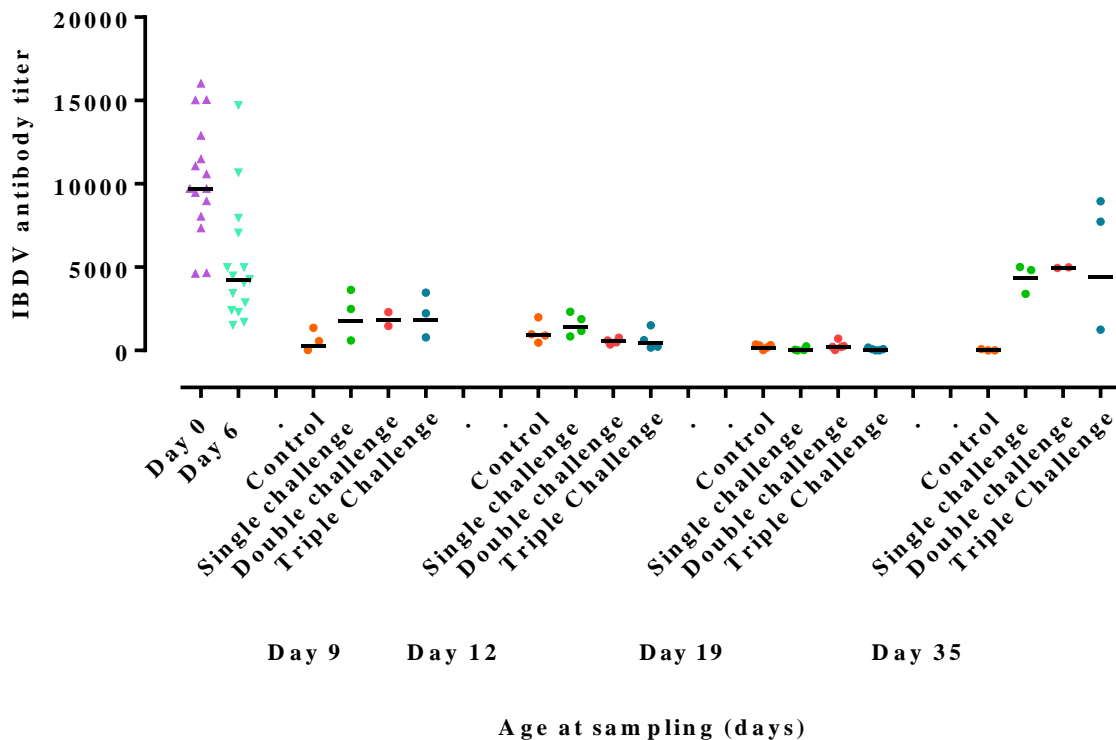


Figure 2-5 Experiment 2; Serum antibody titers to IBDV of the control group and challenged groups. Each data point represents individual titers and horizontal bar indicates the geometric mean.

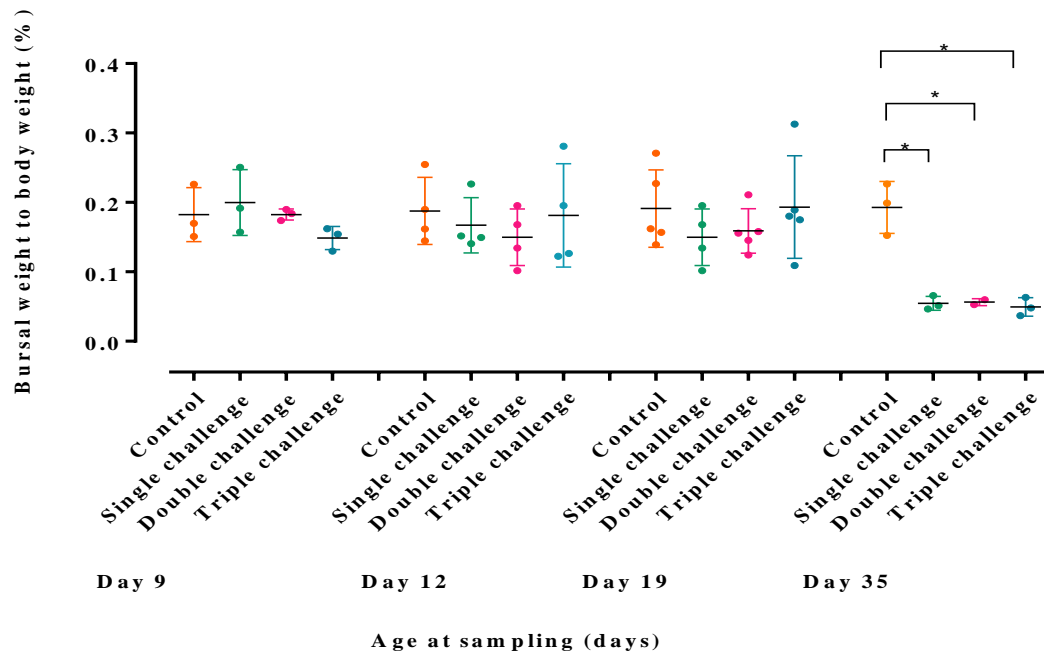


Figure 2-6: Experiment 2; Bursal weight to body weight percentage (BBW) of maternally immune broilers from IBD vaccinated broiler breeders at -9, 12, 19 and 35 d of age. At each age point, 3-5 birds were euthanized from the control group and challenged groups and BBWs were measured. Each data point = individual BBW data, horizontal bar = mean, vertical lines= SD, asterisks and the horizontal brackets= significantly different groups ($P<0.05$).

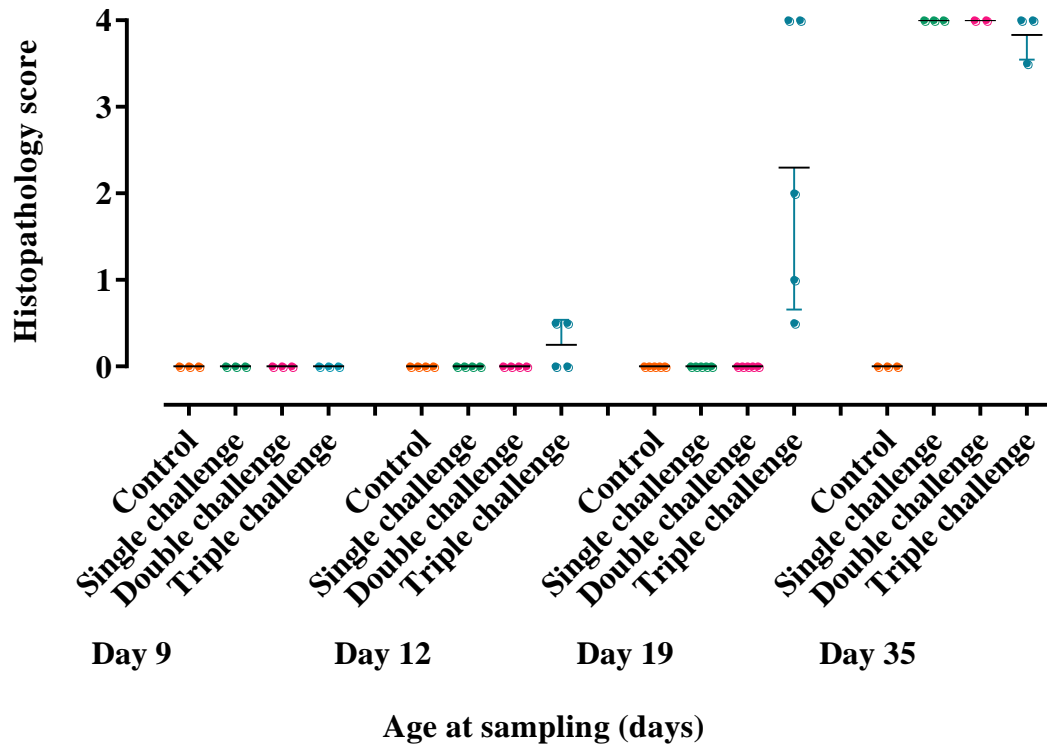


Figure 2-7: Experiment2; Histopathological scores of BF of maternally immune broilers from IBD vaccinated broiler breeders at 9, 12, 19 and 35 d of age. At each age point, 3-5 birds were euthanized from the control group and challenged groups and BFs were samples. The BF were fixed in formalin and histopathological analyze was conducted. The scoring was conducted according to figure 1. Each data point = individual histopathological score, Horizontal bar = mean, vertical lines= SD.

c) Experiment 3: Detrimental effects of single exposure of IBDV variant strain SK09 in maternally non-immune broilers derived from IBD non-vaccinated broiler breeder parents

Day-old chicks had no detectable levels of MAb titers since the parents were not vaccinated. The control group remained antibody negative throughout the study. In contrast, the challenged group had increasing levels of GM (\pm SD) antibody titers at 7, 19 and 35 d of age as follows: 57 ± 154 , 51 ± 2668 and 663 ± 6394 , respectively (Figure 2-8). BBWs of the control birds was significantly higher than that of the challenged group at all the time points ($P < 0.05$) (Figure 2-9).

Further, histopathological lesions indicated the severe lymphoid tissue depletion in BF of challenged birds compared with the unchallenged control birds (Figure 2-10).

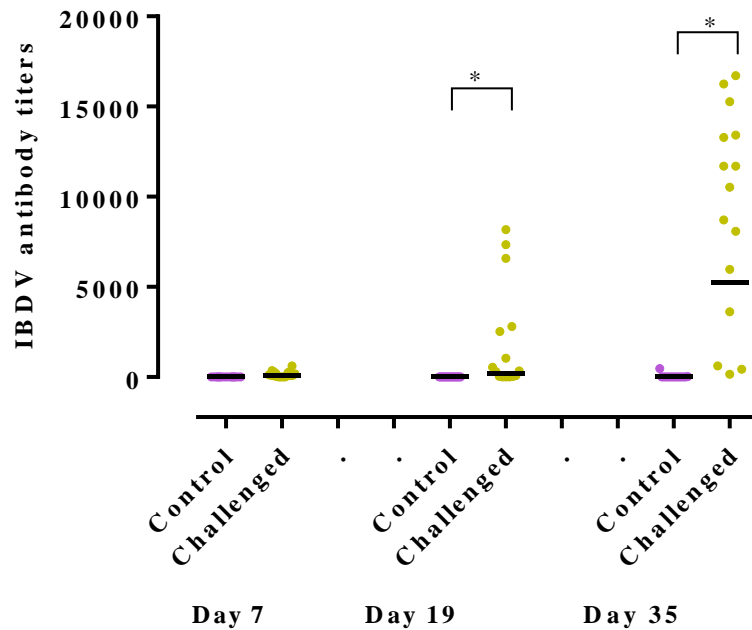


Figure 2-8: Experiment 3; Serum antibody titers to IBV of the control group and one day of age challenged group of maternally non-immune broilers, from broiler breeders who have not been vaccinated for IBV. Each data point represents individual Ab titers and horizontal bar indicates the geometric mean titers. Asterisks and the horizontal brackets= significantly different groups ($P < 0.05$).

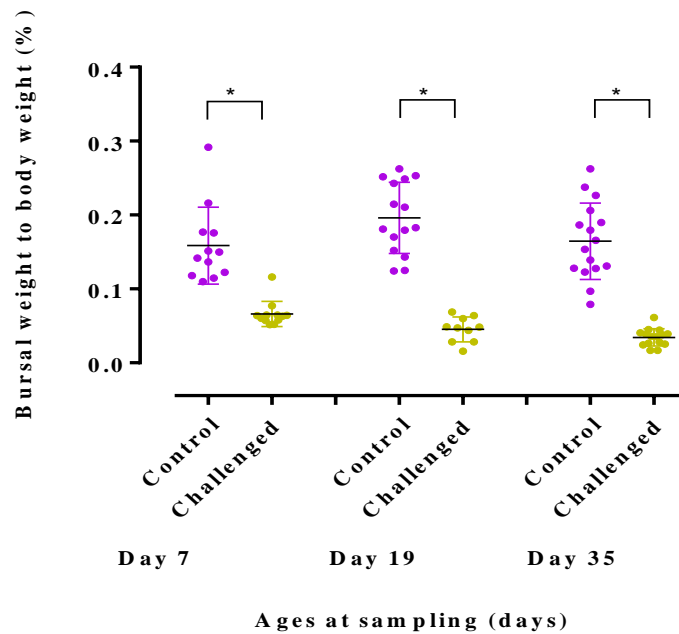


Figure 2-9: Experiment 3; Bursal weight to body weight percentage (BBW) of maternally non-immune broilers from broiler breeders who have not been vaccinated for IBD at days-7 19 and 35 d of age (n=20). Asterisks and the horizontal brackets= significantly different groups ($P < 0.0001$)

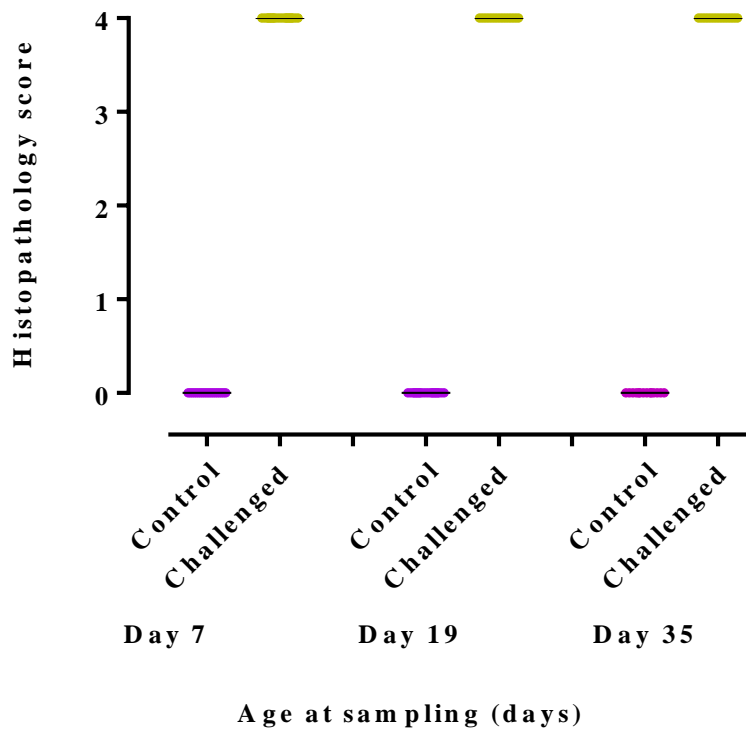


Figure 2-10: Experiment 3 – Histopathological scores of BF of maternally non-immune broilers from broiler breeders who have not been vaccinated for IBD, at 7, 19 and 35 d of age. In every age 20 birds were euthanized from the control group and challenged groups and BBWs were measured. Each data point = individual BBW data, Horizontal bar = mean, vertical lines= SD.

d) Experiment 4: Detrimental effects of single exposure of vIBDV-SK09 in maternally immune broilers derived from IBD vaccinated broiler breeder parents

As expected, the GM (\pm SD) antibody titer of IBDV at hatch was $8\,007 \pm 3456$. The GM (\pm SD) antibody titers of non-challenged control group declined at 7, 19 and 35 d of age as follows; $1\,972 \pm 2418$, 101 ± 313 and 5 ± 16 whereas the challenged group showed an increase in antibody titers as $2\,252 \pm 2040$, 94 ± 231 and $3\,476 \pm 1511$ respectively (Figure 2-11). In spite of the presence of 8 007 mean MAb titer, the challenged group had significantly lower values of BBW at 35 d of age (0.04) compared with the BBW of the control group (0.15) (Figure 2-12). Besides, microscopic

examination of BFs revealed, severe lymphoid atrophy in the challenged birds (Figure 2-13). The mean body weight was also compared to see the effect of vIBDV-SK09 strain to the growth of the broilers. The control group showed significantly higher mean body weight 2 334 g than that of the challenged group 2 242 (P= 0.48)

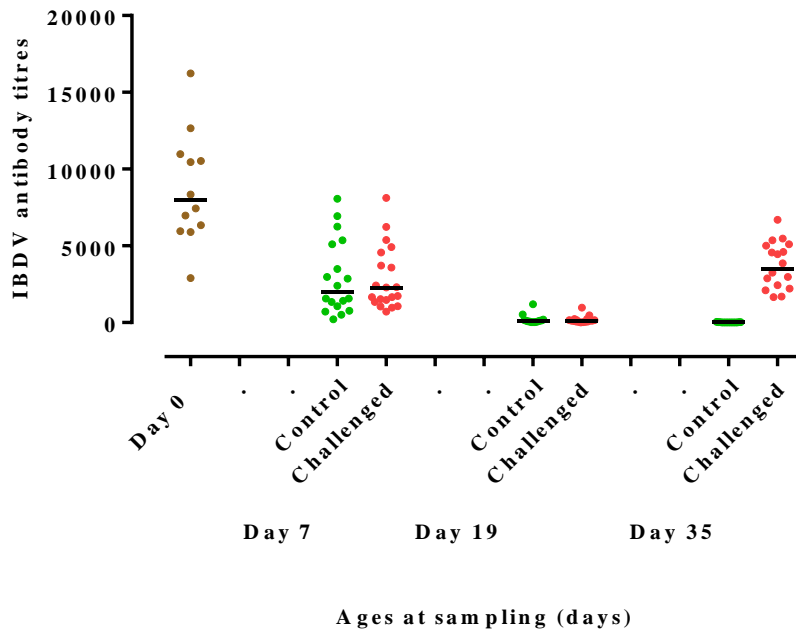


Figure 2-11: Experiment 4 - Serum antibody titers to IBDV of the control group and 1 d of age challenged group of maternally immune broilers from IBD vaccinated broiler breeders. Each data point represents the individual Ab titer. Horizontal bar= geometric mean of Ab titers.

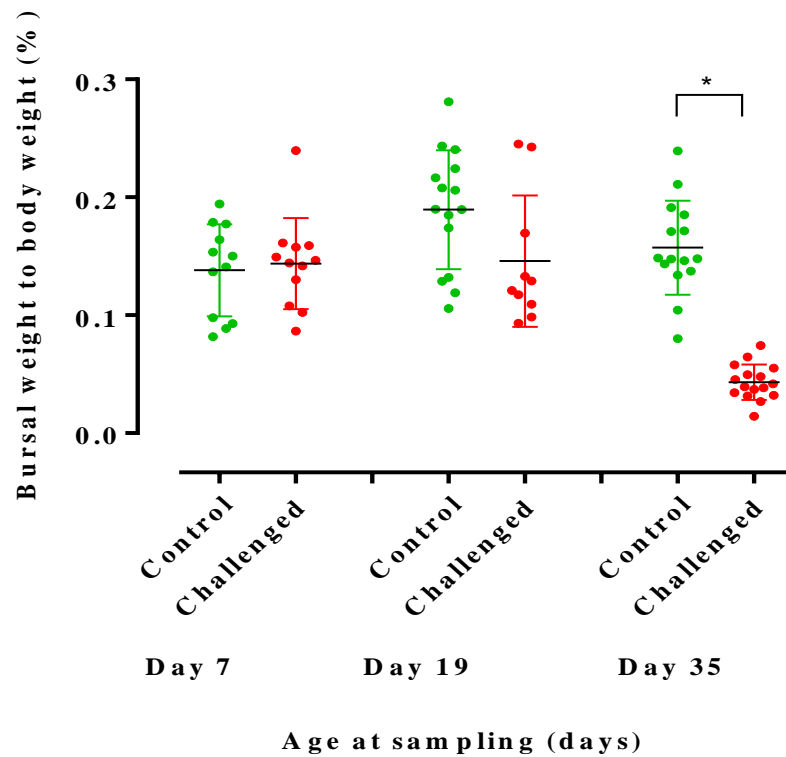


Figure 2-12: Experiment 4, Bursal weight to body weight percentage (BBW) of maternally immune broilers from IBD vaccinated broiler breeders at 7, 19 and 35 d of age. At each time point, 20 birds were euthanized from the control group and challenged groups and BBWs were measured. Each data point = individual BBW data, Horizontal bar = mean, vertical lines= SD. Asterisks and the horizontal brackets= significantly different groups ($P<0.0001$).

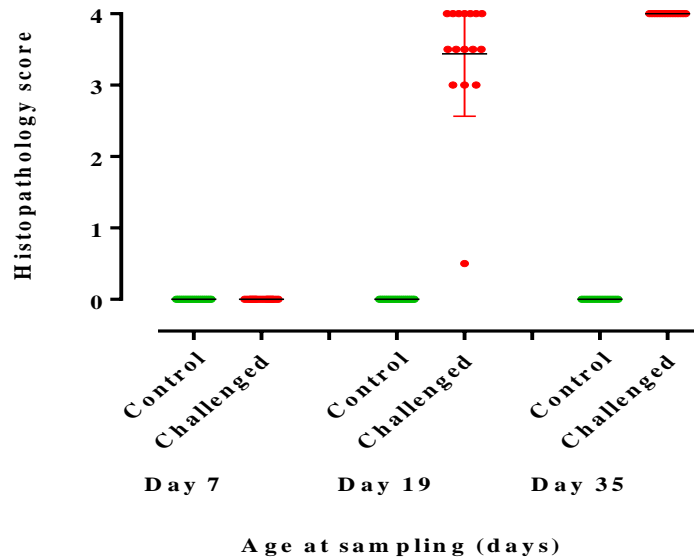


Figure 2.13: Experiment 4 - Histopathological scores of BF of maternally immune broilers from IBD broiler breeders at 7, 19 and 35 d of age. At each time point, 20 birds were euthanized from the control group and challenged groups and BBWs were measured. Each data point = individual BBW data, Horizontal bar = mean, vertical lines= SD.

2.5 Discussion

The field isolate of SK09, a vIBDV characterized in this study, originated from a farm with a history of poor performance. As with many broiler flocks in Canada, the majority of flocks are not vaccinated against IBDV but rely on MAb levels for protection during the first weeks of life. Control of IBDV has been complicated by the recognition of vIBDV in some broiler barns in Canada.

In the current study, we demonstrate the deleterious effects of vIBDV-SK09 in all of the study groups tested. As expected, the SPF leghorns and MAb free broilers showed severe bursal atrophy within less than 6 days following the virus challenge than that of maternally immune broilers. This can be due to the fact that SPF leghorns and MAb free broilers have no specific immunity for any type of IBDV challenge and that can aggravate the pathology of the challenge. It is also important to note, that severe bursal atrophy was observed despite the low challenge dose of IBDV used in experiments 1 and 2, compared with previously reported animal trials (165, 88).

In experiments 1 and 2, there was no difference observed in the severity of the bursal damage between the groups who were challenged one-time vs. multiple times. This observation can be due to the fact that generally single challenge of a live virus can multiply itself and continue the natural multiple exposures to the same bird or to the other by a “rolling reaction” in the flock. In the fourth experiment, the maternally immune broilers who were challenged at 1 day of age with the very high quantity of MAb, also showed severe bursal atrophy at 35 days of age. That provided a strong evidence, that vIBDV-SK09 are able to escape the neutralizing effect of passively transferred MAb from broiler breeder parents and successful infect the BF and cause lymphoid depletion.

The data produced in the current study are compatible with the observations made in an epidemiological study conducted using immunosuppression related broiler samples, presented with higher mortality, poor feed conversion efficiency, and retard meat production had BFs with severe lymphoid depletion, from Saskatchewan broiler farms previously in our lab (239).

In conclusion, it is clearly evident that MAb transferred from hyper-immunized broiler breeder parents who were vaccinated with many combinations of classical IBDV strains, are not enough to control the exposure of vIBDV new strains such as SK09 in broilers. VIBDV-SK09 strain studied here is immunosuppressive not amenable to current vaccines. Therefore, there is an urgent need to identify candidate vaccine strain(s) and a suitable vaccine regimens to prevent and minimize the incidence to vIBDVs in Canadian broiler farms.

PREFACE TO CHAPTER 3

The roots of the current research in my thesis are linked to the epidemiological evidence of novel vIBDV strains circulating in North America. In the USA, Jackwood *et al.*, 2005 demonstrated newly emerging viruses infecting chickens on poultry farms experiencing immunosuppression-related problems. The majority of these vIBDVs were due to the mutations occurring in the VP2 hypervariable region (123). He placed some of these variants in a newly added branches which were not previously identified (123). His work inspired many epidemiological and molecular surveillances of vIBDV in North America. (125) (240). Meanwhile, Canadian broiler farmers were also experiencing mysterious “problem flocks” associated with production losses, clinical signs associated with respiratory and enteric diseases and vaccine failures (129). In 2007, Ojkic *et al.* suggested that many of the above mentioned problems could be associated with immunosuppression caused by vIBDV strains circulating in Canada. He conducted RT-PCR and VP2 gene sequencing studies and revealed the presence of vIBDV in bursal samples collected from “problem broiler flocks” associated with immunosuppression related conditions across four Canadian provinces. vIBDV-SK09 strain is one of the strains that we isolated from Saskatchewan broiler farms experiencing a similar type of immunosuppression related problems (239). The main objective of my research was to demonstrate the immunosuppressive effects of the vIBDV strains, particularly SK09, in a controlled research environment. The first manuscript of the thesis is mainly focused on challenging vIBDV-SK09 in SPF leghorns, MAb free broilers and maternally immune broilers single and multiple times. We were able to produce successful bird models with genetic and immunological variabilities. The immunosuppressive changes are mainly explained through the pathological changes of BF such as reduction of bursal weight in relation to the body weight and microscopically lymphoid depletion. Similar animal trials were conducted using broilers originating from other western Canadian provinces with different broiler breeder vaccination programs against IBDV similar to broilers vaccinated against IBDV in Saskatchewan, Canada (data not shown). None of the vaccination programs showed significant protection against vIBDV-SK09 infection. SPF leghorns and maternally immune broilers were also challenged with 1×10^3 CID₅₀ of fibroblast grown vIBDV-SK09 strain and similar bursal lesions have been demonstrated (data not shown).

In the second manuscript, we intended to move a further step forward by giving a secondary challenged of avian pathogenic *E.coli* following a vIBDV-SK09 challenge. This allowed us to see

how susceptible the birds were for the clinical disease of *E. coli* due to vIBDV-SK09 induced immunosuppression. Importantly, the second manuscript provides valuable data for correlating the clinical significance of immunosuppression with what reported in many epidemiological studies (129) (123).

CHAPTER 3 : IMMUNOSUPPRESSIVE EFFECTS OF VARIANT INFECTIOUS BURSAL DISEASE VIRUS (vIBDV) SK09 IN COMMERCIAL BROILER CHICKENS

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Circulating strains of variant infectious bursal disease virus may pose a challenge for antibiotic-free chicken farming in Canada. Shanika Kurukulasuriya, Khawaja Ashfaq Ahmed, Davor Ojkic, Thushari Gunawardana, Ashish Gupta, Kalhari Goonewardene, Ruwani Karunarathna, Shelly Popowich, Philip Willson, Suresh K. Tikoo, Susantha Gomis*

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3.1 Abstract

Antibiotic-free and safe animal products are most desirable among consumers. However, ensuring safe poultry products is a challenging task when the chicken immune system is compromised. IBDV causes immunosuppression and predisposes chickens to secondary infections. Breeder vaccination against IBDV is routinely practiced for producing chicks with MAb to prevent infection in newly hatched chicks. The majority of IBDV circulating in Canadian farms are vIBDV. Whether circulating vIBDV strains are immunosuppressive in chicks or are amenable to current vaccine regimens has not previously been tested through challenge studies. In this study, day-old broiler chicks (n=240) carrying MAb were obtained from broiler breeders vaccinated with commercial IBDV vaccines. In the first set of experiments (n=40/group), at 6 d of age, one group was challenged with a Canadian field isolate vIBDV -SK09 (3×10^3 EID₅₀). The second and the third groups (controls) were inoculated with non-immunosuppressive IBDV D-78 (10×10^3 TCID₅₀) and saline, respectively. Histopathological examination on days 14 and 30 post-challenge revealed that despite the high level of MAb, vIBDV-SK09 caused severe bursal damage in chicks. Another set of experiments with treatment groups as above, revealed that pre-exposure of chicks with vIBDV-SK09 caused immunosuppression resulting in significantly higher mortality and disease severity in chicks challenged with a virulent strain of *E. coli*. Our data provide evidence that IBDV strains circulating in Canada are immunosuppressive, not amenable to current anti-IBD vaccination strategy and are a potential threat to antibiotic-free chicken farming. Circulating strains of variant infectious bursal disease virus may pose a challenge for antibiotic-free chicken farming in Canada

3.2 Introduction

Environmental and food safety issues have emerged as a major public health concerns worldwide, as animal products may be contaminated with harmful bacteria (241-243). Over several decades, antibiotics have been used as feed additives to mitigate early chick mortality due to bacterial infection in chickens, as well as to ensure bacteria-free and safe products to consumers (244, 245). However, there is growing concern about indiscriminate use of antibiotics in animal production and emergence of antibiotic-resistant strains of bacteria that may eventually adversely affect animal and human health (244-246). There have been several studies about raising antibiotic-free chickens, but all of these studies clearly demonstrated that removal of these drugs leads to poor

production performance and increases the risk of poultry products being contaminated with food-borne illness-causing bacteria (247, 248). In May 2014, Chicken Farmers of Canada voluntarily withdrew use of category 1 antibiotics. However, a recent study conducted in Canadian commercial farms reported that drug-free chicken production leads to poor growth performance and greater incidence of *Clostridium perfringens* and *Campylobacter jejuni* infection in chickens (245). These studies suggest that implementing antibiotic-free poultry farming could be very challenging (247, 245, 248), particularly, if chicken's immune system is compromised by immunosuppression, predisposing chickens to several opportunistic pathogens (249, 250, 245). Therefore, preventing immunosuppression becomes increasingly more important in attempts to minimize the use of antibiotics in poultry production.

In chickens, IBD, also called Gumboro disease, is one the most important immunosuppressive diseases, and creates serious problem for the poultry industry worldwide (137). This disease is caused by IBD virus (IBDV), a highly contagious RNA virus belonging to the family *Birnaviridae* (250). IBDV is classified into two distinct serotypes (*i.e.* serotype I and II). serotype I viruses are pathogenic to chickens and are further classified into classic, variant, and highly virulent strains; whereas serotype II viruses, isolated from turkeys, are apathogenic to chickens (251). IBDV is a non-enveloped virus containing two segments of double stranded RNA (segment A and B) (25). Segment A encodes viral proteins (VPs); VP2, VP3 and VP4; whereas segment B encodes VP1, and VP5 (131). VP2 is the major structural protein responsible for binding to neutralizing antibodies (252). Within the coding region of VP2 a “hyper-variable domain” exists that contains two major hydrophilic regions (252). Substitution mutations in these domains contribute to antigenic drift occurring in the virus, and generate vIBDV.

IBDV in broilers has been controlled by a vaccination strategy to maximize MAb against IBDV by hyper-immunization of broiler breeder parents. A common strategy is to administer a series of live attenuated vaccines followed by an inactivated vaccine “booster” that results in high levels of MAb in the progeny (156). Sometimes, IBDV control measures also combine a broiler vaccination program using attenuated IBDVs (253). Significant economic losses in the poultry industry owing to IBDV-induced immunosuppression have been well documented (254). In the recent past, there has been a significant rise in IBDV infection in Canadian broiler chicken farms associated with production losses, clinical signs associated with respiratory and enteric diseases and vaccine failures (129). Several studies reported that the majority of the IBDV strains circulating

in Canada are variants (130, 129). A recent epidemiological study reported 45 IBDV isolates, 88.89% of the isolates were vIBDV strains, wherein 60% showed high sequence identity to USA isolate vIBDV NC171 and 28.89% were South African 05SA8 strain (130). Besides, a five year epidemiological studies conducted by us on the incidence of IBDV infection in Saskatchewan also revealed that 60%, 20% and 20% of the IBDV isolates were similar to vIBDV strains NC171, Delaware-E and 586, respectively (239). Recently, we found a strong association of IBDV-induced immunosuppression with the rate of condemnation of broiler carcasses in the broiler chicken industry in Saskatchewan (249). Despite several studies in the field, it remained unclear whether circulating vIBDV cause pathogenesis and immunosuppression in chickens; and hence poses a real threat to the poultry industry.

Therefore, the present study investigates the role of circulating vIBDV in causing pathogenesis and immunosuppression in broiler chickens. Challenge experiments were conducted using a Canadian field isolate of IBDV (vIBDV-SK09), which has 98.3% nucleotide sequence identity with NC171), while using non-immunosuppressive IBDV classical strain D78 as a control. The IBDV pathogenesis was studied by measuring the BBW and histopathology of the (BF, whereas the immunosuppression of IBDV infected chickens was evaluated by studying the morbidity and mortality of chickens after an *E. coli* challenge. Results suggest that circulating vIBDV-SK09 is an immunosuppressive virus and is a potential threat to overall poultry health in Canada.

3.3 Materials and methods

3.3.1 Experimental chickens

Day-old broiler chicks were obtained from a local commercial hatchery (Prairie Pride Chick Sales Ltd., Saskatchewan) in Saskatchewan, Canada. The broiler breeder parent flocks of those broiler chicks had been vaccinated against IBDV at 14 d of age (Bursin 2, Zoetis, Kirkland, Quebec), 21 d of age (Bursimune, Ceva Animal Health, Cambridge, ON), 8 weeks of age (Bursa Blen M, Merial, Gainesville, GA), 10 weeks of age (Matimavac) and 18 weeks of age (Maximune Avi-Pro 432 ND-IB2-BD3 REO, Lohmann Animal Health International, Winslow, ME). Birds were maintained in an isolation facility at the ACU, Western College of Veterinary Medicine, University of Saskatchewan. Feed and water were provided *ad libitum*. This work was approved

by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.3.2 Challenge virus and dose

In this study, vIBDV-SK09, isolated from broiler chicken farms in Saskatchewan, Canada, was used as the vIBDV challenge virus. This strain has 98.3% nucleotide sequence identity to vIBDV strain NC171 and was selected to represent circulating vIBDV strains, since a recent epidemiological study demonstrated that the majority of circulating strains in Canada have high sequence identity to NC171 (130). These facts make vIBDV-SK09 a good candidate virus to study the pathogenesis and immunosuppression caused by the currently circulating strains in Canadian chicken farms. Furthermore, we selected non-immunosuppressive, classical IBDV strain D78 (Nobilis Gumboro, Intervet International B.V) as a control challenge for the direct comparison to assess the impact of vIBDV-SK09-mediated immunosuppression on secondary bacterial infection. Challenge virus (vIBDV-SK09) was prepared following standard procedures (136). Briefly, pooled bursal tissue samples collected from vIBDV infected birds were homogenized in PBS to make 40% (w/v) suspensions. The suspensions were centrifuged 3 000 rpm for 10 min and filtered through 45 nm pore size filter. The filtrate was orally administered to 18-day-old SPF chickens (Sunrise Farms, Inc. Catskill, NY). BF were collected 3 d following vIBDV infection and vIBDV was titrated in SPF embryos as described previously (136). In brief, 10-d-old embryonated SPF chicken eggs were inoculated with 0.1ml of filtered (45 nm pore size) bursa homogenate diluted 10-fold in PBS solution (pH 7, containing 10 µg/ml gentamicin sulfate (Gibco, Invitrogen, Carlsbad, CA) via the CAM. The EID₅₀ was determined using the Reed and Munch method (136). For the control challenge study, 3x10³ EID₅₀ viral particles of vIBDV-SK09 and 1x10⁴ TCID₅₀ of IBDV strain D-78 was orally administered per chick.

3.3.3 Bacteria

An *E. coli* field strain isolated from a turkey with septicemia was used as the challenge strain as described previously (242). Briefly, this *E. coli* was nonhemolytic, serum-resistant, serogroup O2; and produced a K1 capsule, aerobactin, and type 1 pili. Aliquots of bacteria in 50% brain heart infusion broth (BHI; Difco, Detroit, MI) supplemented with 25% (w/v) glycerol (VWR Scientific, Inc., Montreal, Quebec, Canada) were stored at -70 °C. For the challenge experiments,

bacteria were cultured for 18–24 h at 37 °C on Columbia sheep blood agar plates (Becton, Dickinson and Company, Maryland, USA). A single colony was added into a 250-ml Erlenmeyer flask containing 100 ml of Luria broth (Difco, Detroit, MI). The culture was grown with shaking at 150 rpm for 16–18 h at 37 °C. After incubation, the stationary phase bacterial culture contained approximately 10^9 colony-forming units (cfu) per ml. The cultures were further diluted with sterile saline to adjust the concentration of bacteria required for challenge experiments (1×10^6 or 1×10^7 cfu/bird) in a volume of 100 ul. Viable bacterial counts were done by plating serial dilutions on Columbia sheep blood agar plates in duplicate, and counting the number of colonies following incubation for 18–24 h at 37 °C.

3.3.4 Experimental design

a) vIBDV-SK09 mediated pathogenesis

The aim of this experiment was to investigate pathology of the BF in commercial broiler chickens caused by vIBDV–SK09 despite the presence of MAb to IBDV. Randomly selected day-old-chicks (n=20) were bled for MAb detection and euthanized. One hundred and twenty day-old-chicks were divided into three groups (n=40). At 6 d of age, group one were given 3×10^3 EID₅₀ viral particles of vIBDV-SK09 inoculum, group two were given 10×10^3 TCID₅₀ D-78, and the third group received 0.1 ml of saline, as the IBDV non-exposed control group. Groups of birds were maintained in three isolation rooms in ACU. At 14 and 30 d pi, sera were collected from 20 birds from each group and birds were euthanized to obtain BBW and histopathology of BF. Sera samples were tested for antibodies against IBDV using a commercial ELISA kit according to manufacturer's instructions (IDEXX, Westbrook, ME). BBW of each bird was determined at necropsy and calculated as bursal weight (g)/body weight (g) x 100 (%). Sections of BF were processed for histopathology by fixing in 10% neutral-buffered-formalin. Fixed tissues were embedded in paraffin and sectioned at 5 µm and stained with H&E. Sections of BF were graded on the basis of lymphoid atrophy as follows: 0 = no visible lesions; 1 = mild, focal to multifocal lymphoid atrophy; 2 = moderate, multifocal lymphoid atrophy; and 3 = severe, diffuse lymphoid atrophy (Figure 3-1).

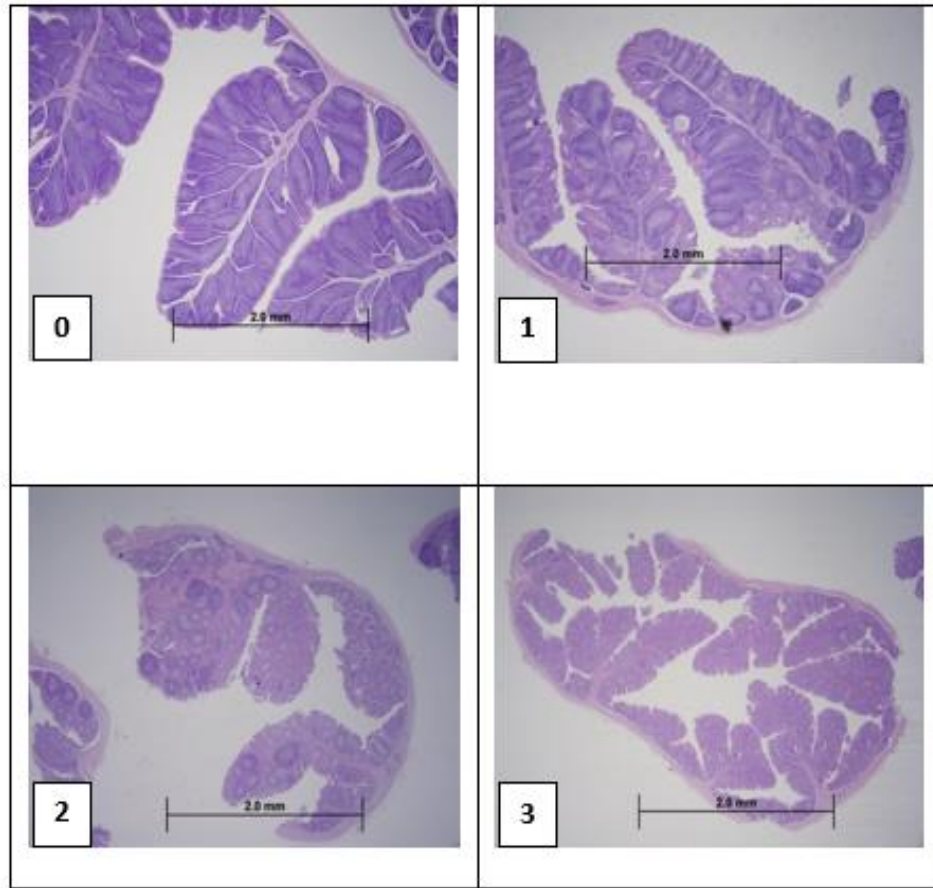


Figure 3-1: Histopathological scoring of BF following exposure of birds to vIBDV. BF was graded on the basis of lymphoid atrophy as follows; 0 = no visible lesions; 1 = mild, focal to multifocal lymphoid atrophy; 2 = moderate, multifocal lymphoid atrophy; and 3 = severe, diffuse lymphoid atrophy.

b) Immunosuppression effects of vIBDV–SK09

The aim of this study was to investigate immunosuppressive effects of vIBDV–SK09 strain in commercial broiler chickens. Three groups of birds each containing 40 birds were maintained at the ACU. One group of birds received 3×10^3 EID₅₀ of vIBDV-SK09 orally at 6 d of age. The second group of birds were given 10×10^3 TCID₅₀ D-78 by the oral route at 6 d of age, which served as non-immunosuppressive IBDV control group. The third group received saline and served as unexposed control. A field isolate of avian pathogenic *E. coli* was used to challenge all three groups at 20 d of age as previously described (255). Briefly, in each group, half of the birds received 1×10^6 (low dose) and other half received 1×10^7 (high dose) cfu of *E. coli* subcutaneously at 20 d of age.

Two doses of *E. coli* were given to groups of birds to simulate field conditions since all birds in a commercial poultry barn are not exposed to a constant dose of *E. coli* (242). Birds were monitored twice daily for 10 d following *E. coli* challenge and each bird was assigned a daily clinical score as follows: 0 = normal; 1 = depressed and hesitant to move; 2 = unable to stand and reach for food or water and 3 = found dead (10). Birds that received a clinical score of 2 were euthanized by cervical dislocation. The clinical score for each bird was summed over the 10 d period and cumulative clinical score (CCS) was calculated as previously described (255). Chickens that were found dead were necropsied immediately and examined for lesions such as pericarditis, perihepatitis, polyserositis and/or any other gross lesions. Bacterial swabs were taken from air sacs of all dead or euthanized birds and cultured on 5% Sheep blood agar and incubated at 37 °C for 24 h. Bacterial colonies were enumerated by scoring on a scale from 0 to 4 as previously described (256) and the animal experiment was terminated 10 d pi.

3.3.5 Statistical analysis

The BBW, histopathological score and antibody titers against IBDV were analysed using the Wilcoxon Rank Sum Test. BBW and CCS values among groups were compared using the Mann-Witney non-parametric test. The homogeneity of distribution of bacterial scores was tested using Fishers exact non-parametric analysis. Survival data were analyzed using Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA) and Statistix7 (Analytical Software, Tallahassee, FL) with a significance level of $P < 0.05$. The survival patterns and median survival times were compared using the log-rank test and chi-square statistic. The relative risk (RR) of mortality for control subjects was calculated using proportional hazards regression.

3.4 Results

3.4.1 Field strain of vIBDV-SK09 is pathogenic in commercial broiler chickens and is not amenable to acquired passive immunity

The geometric mean (GM \pm SD) titer of MAb against IBDV was $7\,834 \pm 3\,576$ at the time of hatch. In the control group, antibodies against IBDV declined to 298 ± 295 and to 25 ± 172 by 19 and 35 d of age, respectively. Similarly, the antibody titer against IBDV was 217 ± 219 and 249 ± 218 at 19 d of age in groups challenged with D-78 and vIBDV-SK09, respectively. At 35 d of

age, the antibody titer against IBDV in the D-78 group was 20 ± 207 ; in contrast the antibody titer against vIBDV-SK09 was $3\ 639 \pm 3\ 569$. ($P < 0.01$) (Table 3-1).

Although there was no significant difference in BBW among three groups at 19 d of age, histopathological examination revealed that birds challenged with vIBDV-SK09 had severe bursal atrophy with a mean lesion score of 2.2 compared to histopathological score of 0 in groups exposed to D-78 or saline (Table 3-1, Figure 3-2). BBW was 0.04 ± 0.01 in the group challenged with vIBDV-SK09 at 35 d of age; in contrast, BBW was 0.14 ± 0.05 and 0.14 ± 0.04 at 35 d of age in groups exposed to saline and D-78 respectively. No histopathological lesions were seen in any of the birds exposed to D-78 or saline at 35 d of age (score = 0) (Table 3-1, Figure 3-2) ($P < 0.05$). The mean body weight of birds in the vIBDV-SK09 group at 35 d of age was $2\ 307 \pm 252$ in contrast, the mean body weight of birds in the D-78 or saline exposed groups at 35 d of age was $2\ 460 \pm 253$ and $2\ 488 \pm 268$ respectively. The vIBDV-SK09 group showed a significant reduction in the body weight compared to the saline group ($P < 0.05$).

Table 3-1: Antibody titer against IBDV, BBW and histopathological score of BF collected at 19 and 35 d of age.

Sampling age (d)	Parameter	Group ^A		
		Saline	D78	SK09
19	Antibody titer ^B	298±295	217±217	249±218
	Bursa to body weight ratio ^C	0.18 ± 0.05	0.16 ± 0.04	0.14 ± 0.06
	Histopathology score ^D	0,0,0,0,0,0,0,0,0 0,0,0,0,0,0,0,0,0	0,0,0,0,0,0,0,0,0 0,0,0,0,0,0,0,0,0	3,3,3,3,3,3,3,3,3,3,3, 1,2,1,1,0,0,0
35	Antibody titer ^B	25(±172) _a	20(±207) _a	3696(±3569) _b
	Bursa to body weight ratio ^C	0.14(±0.05) _c	0.14 (± 0.04) _c	0.04 (± 0.01) _d
	Histopathology score ^D	0,0,0,0,0,0,0,0,0 0,0,0,0,0,0,0,0,0	0,0,0,0,0,0,0,0,0 0,0,0,0,0,0,0,0,0	2,2,2,2,2,2,2,2,2,2, 2,2,2,2,2,2,2

^A Groups of birds were orally administered with vIBDV-SK09 or D-78 at day 6 of post-hatch. Saline Control birds were not exposed to IBDV. Groups whose results are marked with the same lower case sub-script are not different (P>0.05). ^B Antibody titer against IBDV. ^C BBW at 19 and 35 days post-hatch. ^D Histopathology of BF

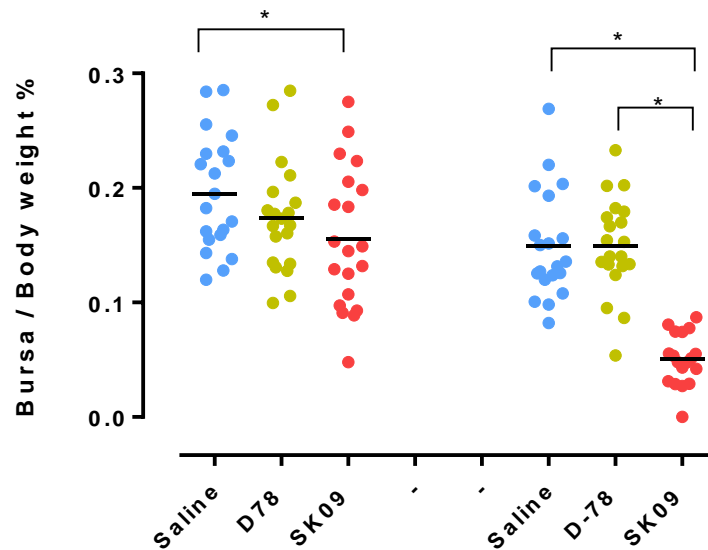


Figure 3-2: BBW of birds at 19 and 35 d of age following D78 and vIBDV-SK09 challenge at 6 d of age. VIBDV-SK09 exposed birds had a significantly lower BBW at 35 d of age). Bar=mean, asterisks and the horizontal brackets= significantly different groups ($P<0.05$)

3.4.2 IBDV-SK09 is immunosuppressive and increases morbidity and mortality in commercial broiler chickens following *E. coli* challenge

The average MAb titer at hatch was $7\,280 \pm 3\,423$. The groups of birds not exposed to vIBDV-SK09 had mortality of 40% following *E. coli* challenge, in contrast, the group exposed to vIBDV-SK09 had significantly higher mortality of 67.5% ($P<0.05$) (Figure 3-3-A). The RR of developing *E. coli* septicemia was determined by comparing the *E. coli* counts in air sacs (Table 3-2). The birds exposed to *E. coli* following vIBDV-SK09 had 1.58 times higher RR of having higher bacterial counts than the group of birds challenged with D78 and *E. coli* ($P<0.05$). The mean CCS in the group of birds not exposed to vIBDV-SK09 was 13 ± 17 , in contrast, CCS of birds exposed to vIBDV-SK09 was 23 ± 22 ($P = 0.054$) (Figure 3-3-B).

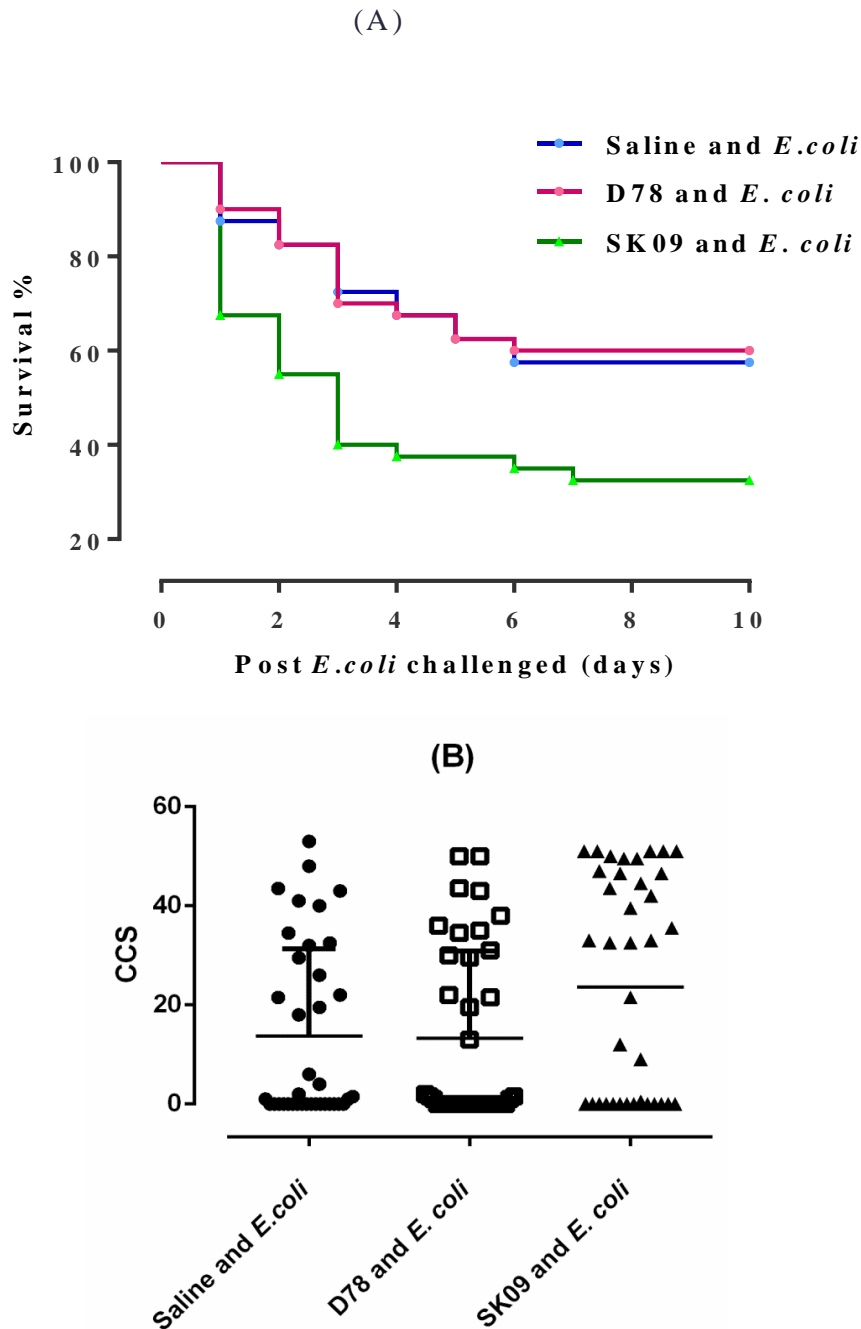


Figure 3-3: Survivability and cumulative clinical score of birds with vIBDV-SK09 following *E. coli* challenge (A) Survival of birds following *E. coli* challenge. Survival of birds exposed to vIBDV-SK09 prior to *E. coli* challenge was significantly lower than birds challenge with D78 and *E. coli* ($P < 0.05$). (B) Cumulative clinical score (CCS) of birds following *E. coli* challenge. The median CCS is higher in birds exposed to vIBDV-SK09 prior to *E. coli* challenge compared to birds challenge D78 and *E. coli* ($P = 0.054$).

Table 3-2: Bacterial isolations from air sacs following *E. coli* challenge (n=40).

Bacterial score	Number of birds		
	<i>E. coli</i> and Saline ^B	<i>E. coli</i> and D78 ^C	<i>E. coli</i> and vIBDV-SK09 ^D
0	23	22	13
1+	3	4	5
2+	5	6	6
3+	3	3	10
4+	6	5	6

^B The group received saline and *E. coli* at day 6 and 20 post-hatch respectively. ^C The group received D78 and *E. coli* at 6 and 20 d of age respectively. ^D The group received vIBDV-SK09 and *E. coli* at 6 and 20 d of age, respectively. The birds exposed to *E. coli* following vIBDV-SK09 had 1.58 times higher RR of having higher bacterial counts than the group of birds challenged D78 and *E. coli* ($P < 0.05$).

3.5 Discussion

Immunosuppression is a problem for the poultry industry worldwide and the economic impacts of immunosuppression can be substantial (257). Several factors like environmental stress, poor nutrition, pathogens and poor management can cause immunosuppression in chickens resulting in increased mortality, decreased body weight, higher feed conversion, uneven growth, and higher rates of condemnation at slaughter (249, 257). Of note, immunosuppression predisposes chickens to respiratory diseases and secondary bacterial infection; indirectly promotes the increasing use of antibiotics and other medications in chicken farming (250). In the meat industry, there is growing concern about excessive use of antibiotics that may contaminate animal products and also lead to the emergence of resistant strains of bacteria (241, 258, 243). These issues have become especially important in the broiler chicken industry in Canada. In May 2014 the chicken industry implemented a policy of antibiotic reduction and responsible use in chicken farms. To implement drug-free chicken farming, the Canadian chicken industry not only needs to pay attention to biosecurity and management practices, but also should reevaluate current disease-prevention strategies (244, 245, 242) as new pathogenic strains are emerging.

It is well-known that when chicks get infected with some strains of IBDV during the critical first 3 weeks of age, chicks can be immunosuppressed, resulting in impaired antibody response, high susceptibility to secondary infections and vaccination failures (250). Studies of IBDV pathogenesis have shown increased susceptibility of chickens to *E. coli* septicemia (259), increased persistence of *Salmonella enteritidis* in intestines (260), and eggs contaminated by SE (261). There is also evidence that avian respiratory viruses, such as Newcastle disease, IB and infectious laryngotracheitis produce a persistent infection when birds had prior exposure to IBDV (262). In addition, IBDV-associated immunosuppression significantly increases mortality due to *Eimeria tenella* parasitic infestation in broiler chickens (263). Recently, we found an increased rate of condemnation of broiler carcasses in the broiler chicken industry in Saskatchewan, Canada due to hepatitis not primarily linked to bacterial infection but strongly associated with anti-IBDV titer and IBDV-induced immunosuppression (249). We hypothesized that due to the IBDV induced immunosuppression, livers of immunosuppressed birds were not able to efficiently clear bacteria and toxins from intestines; hence leading to subacute to chronic hepatitis. Thus, prevention of IBDV-induced immunosuppression is especially important for improving overall chicken disease security and poultry product safety.

The majority of broiler chicken flocks are not vaccinated against IBDV in Canada, but the broiler chicken industry rely on MAb levels for protection against IBDV during the critical initial weeks of the life (130, 129). Control of IBDV has been complicated in recent years by the emergence of variant strains of IBDV in Canada (129). It has been shown that variant strains of IBDV can evade MAb acquired immunity (125, 122). Most strains of IBDV circulating in North America are variant strains. Recent, epidemiological study revealed that many circulating IBDV in Canada have high sequence identity to NC171 (130). However, there is no data available to indicate whether these circulating strains are pathogenic or can cause immunosuppression in chickens.

Therefore, to address these issues, we selected vIBDV-SK09) (98.3% sequence identity to NC171) as a model to study the impact of circulating vIBDV within the broiler chicken industry. First, we wanted to investigate whether vIBDV-SK09 infection can be prevented in chicks by the MAb acquired as passive immunity. Therefore, we obtained chicks from a commercial hatchery, where broiler breeders were hyper-immunized with currently practiced IBDV vaccine regimens. We found that despite high MAb (average titer at hatch was $7\ 280 \pm 3\ 423$) vIBDV-SK09 was

able to inflict severe bursal damage in chicks, resulting in reduced bursal weight and BBW ratio. These data clearly suggest that the vaccination programs currently followed by Canadian chicken industry are not very effective against vIBDV, and reappraisal of vaccination programs is urgently needed to fight against emerging vIBDV strains. This issue is important because previous studies show that vaccine against one variant strain may not give protection against the other variant strains of vIBDV (125, 122). Therefore, it is noteworthy to mention that Canadian vaccination strategy against vIBDV should be designed based on the knowledge of circulating strains of vIBDV in Canada.

We next investigated if vIBDV-SK09 can induce immunosuppression in chicks leading to exacerbation of bacterial infection. Several bacterial infections of chicken like, *Salmonella*, *E. coli*, *C. perfringens* and *C. jejuni* have zoonotic potential (245). However, in the present study, we used a well-studied *E. coli* challenge model to assess the impact of immunosuppression on secondary bacterial infection in chicken (255, 242). To compare immunosuppression, we selected IBDV (strain D78) as a control IBDV, which is a well-known non-immunosuppressive live virus, used as vaccine. Fourteen days post IBDV exposure, when chicks were challenged with *E. coli*, we found increased mortality in those broiler chickens that were exposed to vIBDV-SK09 prior to *E. coli* infection. Birds exposed to vIBDV-SK09 experienced 1.6 times higher risk of developing *E. coli* septicemia compared to broiler chickens exposed to D78. The higher bacterial loads in air sacs of the group pre-exposed to vIBDV-SK09 further supports the immunosuppressive role of vIBDV-SK09 to reduce bacterial clearance and increase susceptibility to *E. coli* septicemia.

In conclusion, this study of vIBDV-SK09 suggests that circulating strains of vIBDV are pathogenic and are difficult to control with the available vaccines. The Canadian chicken industry needs an improved anti-IBDV strategy in order to deal with circulating vIBDV strains. In particular, the immunosuppression caused by these circulating IBDV strains is the biggest challenge for the Canadian chicken industry for enhancing the disease security in poultry as well as ensuring the food safety and human health.

PREFACE TO CHAPTER 4

As stated in the third chapter we have demonstrated that vIBDV circulating in Canada were not neutralized by passively transferring MAb from hyper-immunized broiler breeder parents. In fact, the challenged vIBDV-SK09 was successfully escaped the maternal antibodies and caused a severe bursal damage which ultimately lead to increase susceptibility to *E. coli* infections. The overall objective of the first and second chapters were to evaluate the efficacy of current broiler breeder vaccines against vIBDV-SK09. In the fourth chapter our objective was to study effect of broiler vaccines against vIBDV. Broiler vaccination programs are designed to protect broilers against IBDV when MAb decline with their growth. Countries with endemic vvIBDV infection have reported that these broiler vaccines are efficient method of controlling vvIBDV however, the efficacy of broiler vaccines against vIBDV has not been studied in Canada. In order to study efficacy of broiler vaccines against vIBDV, we have studied the efficacy of a live attenuated and a vectored vaccines against IBDV commonly use in the broiler chicken industry in Canada.

CHAPTER 4 : MODIFIED LIVE INFECTIOUS BURSAL DISEASE VIRUS (IBDV) VACCINE RATHER THAN HERPESVIRUS TURKEY (HVT)-IBDV VECTORED VACCINE DELAYS VARIANT IBDV PATHOGENESIS DURING EARLY AGE INFECTION IN BROILERS

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This work has been accepted by *Vaccine*. Modified live infectious bursal disease virus (IBDV) vaccine delays infection of neonatal broiler chickens with variant IBDV compared to turkey herpesvirus (HVT)-IBDV vectored vaccine
Shanika Kurukulasuriya, Khawaja Ashfaque Ahmed, Davor Ojkic, Thushari Gunawardana, Betty Chow Lockerbie, Shelly Popowich, Philip Willson Suresh K. Tikoo, Susantha Gomis*

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4.1 Abstract

Chickens are commonly processed around 35-45 days of age in broiler chicken industry hence; diseases that occur at a young age are of paramount economic importance. Early age infection with IBDV results in long-lasting immunosuppression and profound economic losses. To our knowledge, this is the first study comparing the protection efficacy of MLV of IBDV and HVT-IBDV vaccines against early age vIBDV infections in chicks. Experiments were carried out in IBDV MAb positive chicks (n=330), divided into 6 groups (n=50-60/group), namely Group 1 (saline), Group 2 (saline + vIBDV), Group 3 (HVT-IBDV), Group 4 (HVT-IBDV + vIBDV), Group 5 (MLV) and Group 6 (MLV + vIBDV). HVT-IBDV vaccination was given via the *in-ovo* route to 18 d old embryonated eggs. MLV was administered via the subcutaneous route in day-old broilers. Group 2, Group 4 and Group 5 were orally challenged with vIBDV (SK-09, 3×10^3 EID₅₀) at 6 d of age. IBDV seroconversion, BBW and bursal histopathology were assessed at 19 and 35 d of age. Histopathological examination at 19 d of age revealed that vIBDV-SK09 challenge caused severe bursal atrophy and lower BBW in HVT-IBDV but not in MLV vaccinated chicks. However by 35 d of age, all challenged groups showed bursal atrophy and seroconversion. Interestingly, qRT-PCR analysis after vIBDV-SK09 challenge demonstrated an early (9 d of age) and significantly high viral load (~ 5744 folds) in HVT-IBDV vaccinated group vs unvaccinated challenged group (~ 2.25 folds). Furthermore, flow cytometry analysis revealed inhibition of cytotoxic CD8⁺ T-cell response (CD44-downregulation) and decreased splenic lymphocytes counts in chicks after HVT-IBDV vaccination. Overall, our data suggest that MLV delays vIBDV pathogenesis, whereas, HVT-IBDV vaccine is potentially immunosuppressive, which may increase the risk of early age vIBDV infection in broilers.

4.2 Introduction

IBDV belongs to the *Birnaviridae* family, which is a leading cause of immunosuppression in poultry. Early IBDV infection in chicks less than 3 weeks of age causes subclinical infection, permanent bursal damage, resulting in long-lasting immunosuppression and tremendous economic losses due to vaccine failures and increased susceptibility to a variety of opportunistic pathogens (90). IBDV is highly resistant to many disinfectants and is very difficult to remove from the contaminated poultry premises (7). IBDV is a non-enveloped RNA virus comprising two segments of double stranded RNA (segment A and B) (25). VPs; VP2, VP3 and VP4 are encoded by segment

A; whereas segment B encodes VP1, and VP5 (131). VP2 is the major structural protein responsible for binding to neutralizing antibodies (252). VP2 is considered as the foremost immunogenic component of the virus and thus being targeted for vaccine production for a protective immunity to the virus (264). However, it has also been suggested that VP2 is not the sole determinant of virulence (151). The ability of VP1 altering replication efficacy may play a vital role in determining virulence (148).

IBDV in broilers has been controlled by hyper-immunization of broiler breeder parents, wherein chicks develop passive immunity through acquired MAb (265). In newly hatched chickens, MAb specific to IBDV show a curvilinear decline with age that potentially increases the susceptibility to IBDV infection (123, 266). The emergence of vIBDV (267) and vvIBDV strains (268) which escape MAb resulted in changes in vaccine regimens. Therefore, in addition to hyper-immunization of broiler breeders, broiler vaccination is also being practiced to improve the immunity of the birds against IBDV (269, 270). MLVs have been introduced to the industry and classified as mild, intermediate, intermediate plus and hot IBD vaccines are therefore based on the intensity of virulence. The intermediate and hot MLVs are better in protection but there is a risk that vaccine virus itself can cause bursal damage (271) (205). Nonetheless, MLV vaccination by the subcutaneous route has been shown to be safe without causing bursal damage (272). The recombinant vector vaccines was a remarkable accomplishment in vaccine production that combined safety and efficacy in the presence of MAb. HVT has been widely used in conventional vaccination against Marek's disease (MD) (217). HVT was used to develop a vaccine against IBD (221). Since then, several HVT-IBDV-VP2 vector vaccines have been developed for *in-ovo* or subcutaneous vaccination (223, 222, 220, 224). HVT vector vaccines are known to induce both humoral and cell-mediated immunity with a long lasting protection (220). However, most of the studies showing protective efficacy of HVT-IBDV have challenged birds with pathogenic IBDV at 18 or 28 d (273) or later (274) after immunization. It is not known if a HVT-IBDV vaccine can be protective if chicks are infected during the critical first week of their life.

Use of conventional vaccines prepared with cIBDV, along with strict bio-security measures was successful in controlling IBDV infection until the emergence of antigenically variant strains (vIBDV) (133) and highly pathogenic very virulent IBDV (vvIBDV) (275). Epidemiological surveys and phylogenetic analysis revealed that the majority of the IBDV strains circulating in Canada are variants (130, 129), wherein about 60% of IBD cases were linked to vIBDV strains

(130) and associated with production losses, vaccine failures, clinical signs involving respiratory and enteric diseases (129). Recently, we found that a circulating vIBDV strain (isolate vIBDV-SK09) is immunosuppressive, which can establish infections in chickens despite high levels of MAb (276). Recent studies in layer (277) and broiler (278) chickens compared HVT-IBDV (subcutaneous or *in ovo* route) and MLV (oral or *in ovo* route) vaccines and suggested that HVT-IBDV is superior to the MLV vaccine. Most studies comparing the efficacy of HVT-IBDV and MLV vaccines were focused on the clinical form of the disease (IBDV infections during 3-6 weeks of age) (279-281). However, efficacy against subclinical vIBDV infections that occurs in young age chicks during the critical first week of life remain elusive.

In this study, we investigated whether HVT-IBD or MLV vaccines can provide protection against IBDV, if young chicks acquire circulating vIBDV infection at 1 week of age. This issue is also relevant given that the HVT vaccine has been shown to suppress antigen-specific lymphoproliferative responses (282) causing mild immunosuppression by decreasing leucocytes and lymphocytes number during the first week after vaccination (283). Results suggest that none of the vaccines (HVT-IBD and MLV) tested in the present study provided full protection against vIBDV. However, the MLV, but not the HVT-IBD vaccine, significantly delayed vIBDV pathogenesis and viral replication in young chicks. Remarkably, HVT-IBD vaccination induced immunosuppression in young chicks facilitating vIBDV replication.

4.3 Materials and methods

4.3.1 Experimental chickens

Broiler hatching eggs were obtained from a local hatchery (Prairie Pride Chick Sales Ltd., Saskatchewan, Canada), whose parents follow routine hyper-immunization (276). Birds were hatched and maintained in an isolation facility at the ACU, Western College of Veterinary Medicine, University of Saskatchewan. Feed and water were provided *ad libitum*. This study was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

4.3.2 Vaccines

This study was conducted to assess the efficacy of two commercially available vaccines against early age vIBDV infection. A MLV, Univax-BD (Merck Animal Health, Intervet Inc.,

Kirkland, QC), and a recombinant HVT-IBD vectored vaccine, Vaxxitek® (Merial Canada Inc, Baie-D'Urfe, QC), carrying the VP2 gene of the classical Faragher 52/70 IBDV strain (284), were used.

4.3.3 Challenge virus

A bursal derived field isolate vIBDV SK09 strain was used as the challenge virus (276). The BF were pooled, homogenized, centrifuged and the filtrate was orally administered to 18-day-old SPF leghorn chickens (Sunrise Farms, Inc. Catskill, NY). BF were collected 3 d pi and virus titers were determined by inoculating 10 d old embryonated SPF chicken eggs via the CAM route (136). The EID₅₀ was determined using Reed and Munch method (136).

4.3.4 Monoclonal antibodies

Monoclonal antibodies against chicken CD3, CD4 and CD8 were purchased from Southern Biotechnology Associates (Birmingham, Ala, USA). Mouse anti-chicken CD44 monoclonal antibody was purchased from Bio-Rad (Raleigh, NC, USA). Goat anti-mouse IgG, Streptavidin-PerCP/Cy5.5 and Mouse IgG1 isotype control was purchased from BioLegend (San Diego, CA, USA).

4.3.5 Experimental design - investigation on HVT-IBDV and MLV vaccines efficacy in the prevention of early age IBDV infection

Broiler hatching eggs from broiler breeders vaccinated with commercial IBDV vaccines were used to obtain day-old broiler chicks (n=330) carrying MAb against IBDV. Experiments were carried out using 6 groups, Group 1 (saline control, n=60), Group 2 (saline + vIBDV, n=50), Group 3 (HVT-IBDV, n=60), Group 4 (HVT-IBDV + vIBDV, n=50), Group 5 (MLV, n=60) and Group 6 (MLV + vIBDV, n=50). In the HVT-IBDV vaccinated group, 18 d old embryonated eggs were vaccinated with HVT-IBDV by the *in-ovo* route according to the manufacturer guidelines. Each egg received a full dose (50 µl) of the vaccine through the amniotic cavity using 22 gauge x 1 in needles, followed by sealing. Control eggs received 50 µl saline. In MLV groups, commercially available MLV Univax-BD (Merck Animal Health, Intervet Inc., Kirkland, QC) was used to vaccinate the day-old broilers. of IBD was used to vaccinate the one-day-old broilers with MAb. Each bird received one dose (200 µl) of vaccine. Group 2, Group 4 and Group 5 were orally challenged with vIBDV-SK09 (3×10^3 EID₅₀) at 6 d of age (276). IBDV seroconversion, BBW

and bursal histopathology were assessed at 19 and 35 d of age. Sera were tested for antibodies against IBDV using a commercial ELISA kits according to manufacturer's instructions (PROFLOCK® Plus, Synbiotics Corp, San Diego, CA). BBW of each bird was determined at necropsy and calculated as bursal weight (grams)/body weight (grams) x 100. Sections of BF were processed for histopathology by fixing in 10% neutral-buffered-formalin. Formalin fixed tissues were embedded in paraffin and sectioned at 5 µm and stained with H&E. Sections of BF were graded on the basis of lymphoid atrophy as follows; 0 = no visible lesions; 1 = mild, focal to multifocal lymphoid atrophy; 2 = moderate, multifocal lymphoid atrophy; and 3 = severe, diffuse lymphoid atrophy. In addition, bursal tissue (n=5) were collected from all groups at 9, 20 and 35 d of age for quantifying relative viral loads. Spleen samples (8-10/group) were collected from Group 1, Group 3 and Group 5 for flow cytometric analysis of lymphocyte population to investigate CD4⁺ and CD8⁺ T-cells as well as CD8⁺ T-cell activation at 7 days of age.

4.3.6 RNA isolation and reverse transcriptase-polymerase chain reaction

Following vIBDV SK09 challenge (at 6 d of age), the bursal samples (n=5) were collected at 9, 19 and 35 d of age. About 5 mg of tissues were used for extracting total RNA using RNeasy mini kit (Qiagen, Mississauga, ON) following the manufacturers protocol. Total RNA from each sample was dissolved in 50 µl of RNase-free water. RNA quality was determined by agarose gel electrophoresis. RNA was quantified spectrophotometrically using NanoDrop 2000 (Thermo Fisher Scientific). First-strand cDNA was synthesized using 1 µg of total RNA, Moloney Murine Leukemia Virus Reverse Transcriptase and primers (oligo-dT and random hexamer mixed) in 20 µl volume, according to manufacturer's protocol. All cDNA samples were stored in -80 °C until further use.

4.3.7 Real-time PCR analysis for the quantification of IBDV viral load in bursal tissue

Viral load quantification in bursal tissue was determined using real-time PCR (Mx3000P qPCR system, Agilent Technologies) and TaqMan probes (285). The primers to amplify a 74 bp fragment of IBDV VP2 gene were forward primer 5'-GGACACAGGGTCAGGGTCAAT-3' (VP2-F) and reverse primer 5'-GCAGTGTGTAGTGAGCACCCA-3' (VP2-R). The TaqMan probe used to identify 74 bp VP2 fragment was 5'-TCTTTTCCCTGGATTCCCTGGCTCA-3',

which was labelled with FAM (reporter dye) and ZEN/IBFQ (double quencher) at 5' and 3', respectively. To quantify relative abundance, chicken 18S rRNA was used as housekeeping gene to normalize the quantity of cDNA in PCR reactions. TaqMan primers used to amplify a 186 bp fragment of chicken 18S rRNA were as follows, forward primer 5'-CGGCTACCACATCCAAGGAA-3' (18S-F) and reverse primer 5'-GCTGGAATTACCGCGGCT-3' (18S-R). The TaqMan probe used to detect 18S rRNA target was 5'-TGCTGGCACCAGACTTGCCCTC-3', which was labelled with HEX reporter dye at 5' and ZEN/IBFQ double quencher at 3'. The IBDV VP2 and 18S RNA amplifications were performed in the same tube (20 µl total volume) using Prime Time-Gene Expression Master Mix (IDT) and 2 µl of cDNA template. The PCR amplification conditions were, initial melting at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 s at 60 °C. Serially diluted cDNA samples were used to determine PCR efficiency. Real-time PCR data were expressed as threshold cycle (Ct) value, that is, the number of cycle of PCR at which the fluorescence emitted by the reporter dye crosses a threshold fluorescence value as determined by the software of PCR machine. The lower Ct value suggests that fluorescence intensity of reporter dye passed threshold early indicating higher the initial amount of DNA of interest. For real-time PCR, samples were used in triplicate, maintaining no template control (NTC), IBDV negative and IBDV positive samples in each experiment. IBDV viral loads in bursal tissues were determined by comparative Ct method ($\Delta\Delta C_t$ method) by first normalizing data for differences in the amount of RNA among samples using 18S rRNA Ct value of each sample. Ct value of VP2 NTC and naïve samples were either not detected or were around 39. Therefore, naïve control Ct value was set 39 in calculation. Following formula was used to estimate comparative viral load in bursal tissues:

$$\Delta C_t (\text{test sample}) = C_t \text{ value of VP2 (test sample)} - C_t \text{ value of 18S sRNA (test sample)}$$

$$\Delta C_t (\text{naïve control}) = C_t \text{ value of VP2 (naïve control)} - C_t \text{ value of 18S sRNA (naïve control)}$$

$$\Delta\Delta C_t = \Delta C_t (\text{test sample}) - \Delta C_t (\text{naïve control})$$

$$\text{Fold} = 2^{-\Delta\Delta C_t}$$

4.3.8 Fluorescence-activated cell sorting (FACS) analysis

At 7 d of age, spleen samples from 8-10 chickens per group were collected in unimmunized control and vaccinated groups (MLV and HVT-IBDV). Cell preparation and FACS staining was done as previously described with some modifications (286). Spleens were excised, and single-

cell suspensions were separately prepared by crushing the organs using steel mesh and rubber head of syringe plunger. Lymphocytes were separated using Histopaque-1077 (Sigma-Aldrich, Oakville, ON, Canada). For three-color staining, 5×10^5 cells were incubated with anti-chicken CD3, CD4, and CD8 monoclonal antibodies at 4 C for 30 min, washed three times with FACS wash buffer (PBS containing 2% fetal bovine serum and 0.1% sodium azide). Then, cells were stained with Streptavidin-PerCP/Cy5.5 at 4 C for 30 min for labelling CD3⁺ cells. Following incubation cells were washed three-times before flow cytometric analysis. For two-color staining, 5×10^5 cells were incubated with either unlabeled mouse anti-chicken CD44 or mouse-IgG1 isotype control for 30 min at 4 C. Following there washes with FACS wash buffer, cells were stained with anti-mouse IgG-PerCP/Cy5.5 at 4 C for 30 min. After three washes, cells were stained with anti-chicken CD8 at 4 C for 30 min, subsequently washed three times and resuspended in FACS buffer for the analysis. Flow cytometry data were acquired by EpicsXL (Beckman Coulter) and FACSCaliber (BD Bioscience), and data analyzed with FlowJo software (TreeStar).

4.3.9 Statistical analysis

The BBW, histopathological score, and antibody titer against IBDV were analyzed using Wilcoxon Rank Sum Test (to compare 2 groups) or Kruskal–Wallis One-way ANOVA (to compare more than 2 groups). Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA) and Statistix7 (Analytical Software, Tallahassee, FL) was used for all the analysis with a significance level of $P < 0.05$.

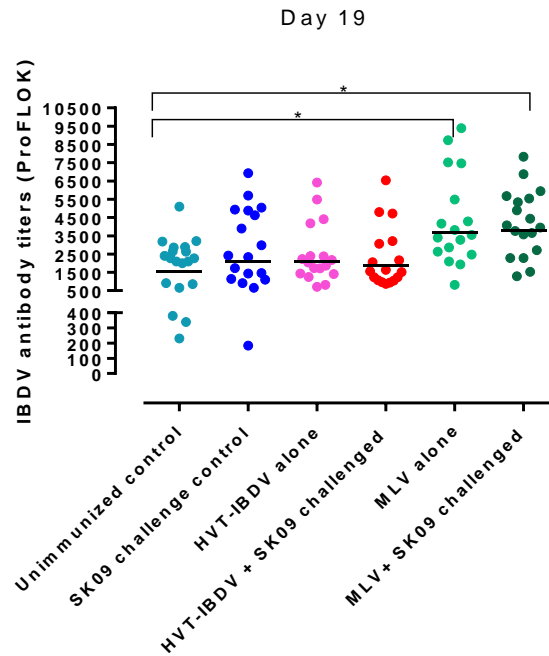
4.4 Results

4.4.1 IBDV antibody titers

Day old birds had a mean MAb titer of 8 144 ($\pm 3 423$) at the time of hatch. IBDV specific MAb levels declined overtime, as evidenced by the IBDV antibody titer at 19 d (Figure 4-1A) and 35 d of age (Figure 4-1B) in the unvaccinated control group. At 19 d of age mean, antibody titers in HVT-IBDV ($2 507 \pm 1 653$) and HVT-IBDV+ vIBDV-SK09 challenged ($2 272 \pm 1 661$) groups were not significantly different than MAb carrying unvaccinated controls ($2 055 \pm 1 245$) (Figure 4-1A). In contrast, MLV alone ($4 347 \pm 2 508$) and MLV + vIBDV-SK09 challenged ($4 200 \pm 1 802$) groups had significantly ($P < 0.05$) higher anti-IBDV titer (19 d) compared to MAb carrying unvaccinated controls ($2 055 \pm 1 245$) (Figure 4-1A). Moreover, the anti-IBDV titer of the MLV + vIBDV-SK09

challenged ($4\,200 \pm 1\,802$) group was significantly ($P < 0.05$) higher than the HVT-IBDV+ vIBDV-SK09 challenged ($2\,272 \pm 1\,661$) group (Figure 4-1A). At 35 d of age, mean antibody titers in the HVT-IBDV alone ($1\,031 \pm 972$) and the MLV alone ($1\,256 \pm 1\,412$) groups were not significantly different than MAb carrying unvaccinated controls (247 ± 204) (Figure 4- 2B). However, there was a remarkable ($P < 0.05$) sero-conversion by 35 d of age in vIBDV-SK09 challenged alone ($9\,883 \pm 5\,146$), HVT-IBDV + vIBDV-SK09 challenged ($7\,714 \pm 5\,616$) and MLV + vIBDV-SK09 challenged ($8\,921 \pm 2\,269$) groups (Figure 4-1B).

A



B

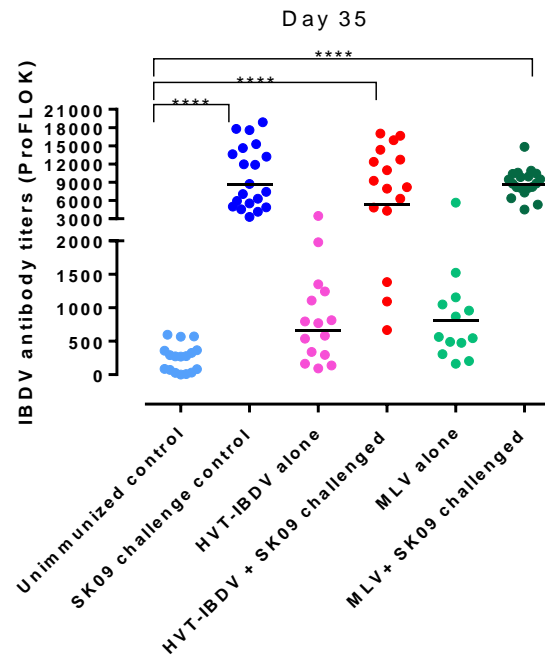


Figure 4-1 : Antibody titer (log10of unimmunized (saline) or vIBDV-SK09 challenged or HVT-IBDV vaccinated or HVT-IBDV + vIBDV-SK09 or MLV vaccinated or MLV + vIBDV-SK09 chickens . HVT-IBDV (Vaxxitek) vaccination via the *ino-ovo* route (18 d embryo). MLV (Univax-BD) subcutaneously route day-old chicks. Serum collected at 9, 19 and 35 d of age. Data presented as geometric mean. Asterisks and the horizontal brackets= significantly different groups ($P<0.05$)

4.4.2 Bursal weight/Body weight ratios, gross lesions and histopathology of BF

Unchallenged groups (negative control and vaccine alone groups) had no evidence of reduction in size of BF and microscopic lymphoid depletions throughout the trial. At 19 and 35 d of age, vIBDV-SK09 challenge alone and HVT + vIBDV-SK09 challenge groups showed significantly ($P<0.05$) lower BBW values compared with the unchallenged control group (0.20 ± 0.04 and 0.17 ± 0.05 , respectively) (Figure 4-2A). The mean BBW values at 19 and 35 d of age of the rHVT + vIBDV-SK-09 challenge group were (0.09 ± 0.04) and 0.04 ± 0.02 , respectively (Figure 4- 2A). The above mentioned values had no statistical difference when compared with the challenge alone group at corresponding days (0.07 ± 0.02 and 0.05 ± 0.01 , respectively). HVT + vIBDV-SK09 challenge and vIBDV-SK09 challenge alone groups produced significantly ($P<0.05$) higher histopathological scores at 19 and 35 d of age compared with the unchallenged groups (negative control and HVT alone) (Figure 4- 2B). High histopathological scores following the vIBDV-SK09 challenge indicated severe bursal lymphoid depletion caused by successful infection of challenged virus in bursa lymphocytes. In contrast, no indication of bursal atrophy or bursal lymphoid depletions were observed on histopathological examination at 19 d of age in the MLV and MLV+ challenge groups (Figure 4-2B), which is further supported by no appreciable change in the BBW ratio of MLV (0.17 ± 0.04) and MLV+ challenge (0.16 ± 0.04) groups (Figure 4- 2A). However, at 35 d of age, there were significantly ($P<0.05$) lower mean BBW values (Figure 4-2A) and higher bursal scores (Figure 4-2B) in MLV + vIBDV-SK09 challenged (0.05 ± 0.01) group compared with the negative control (0.17 ± 0.05) or MLV alone group (0.18 ± 0.06). Higher bursal scores as noted at 35 d of age in the vIBDV-SK09 challenged groups suggesting severe bursal lymphoid depletion caused by the challenged virus.

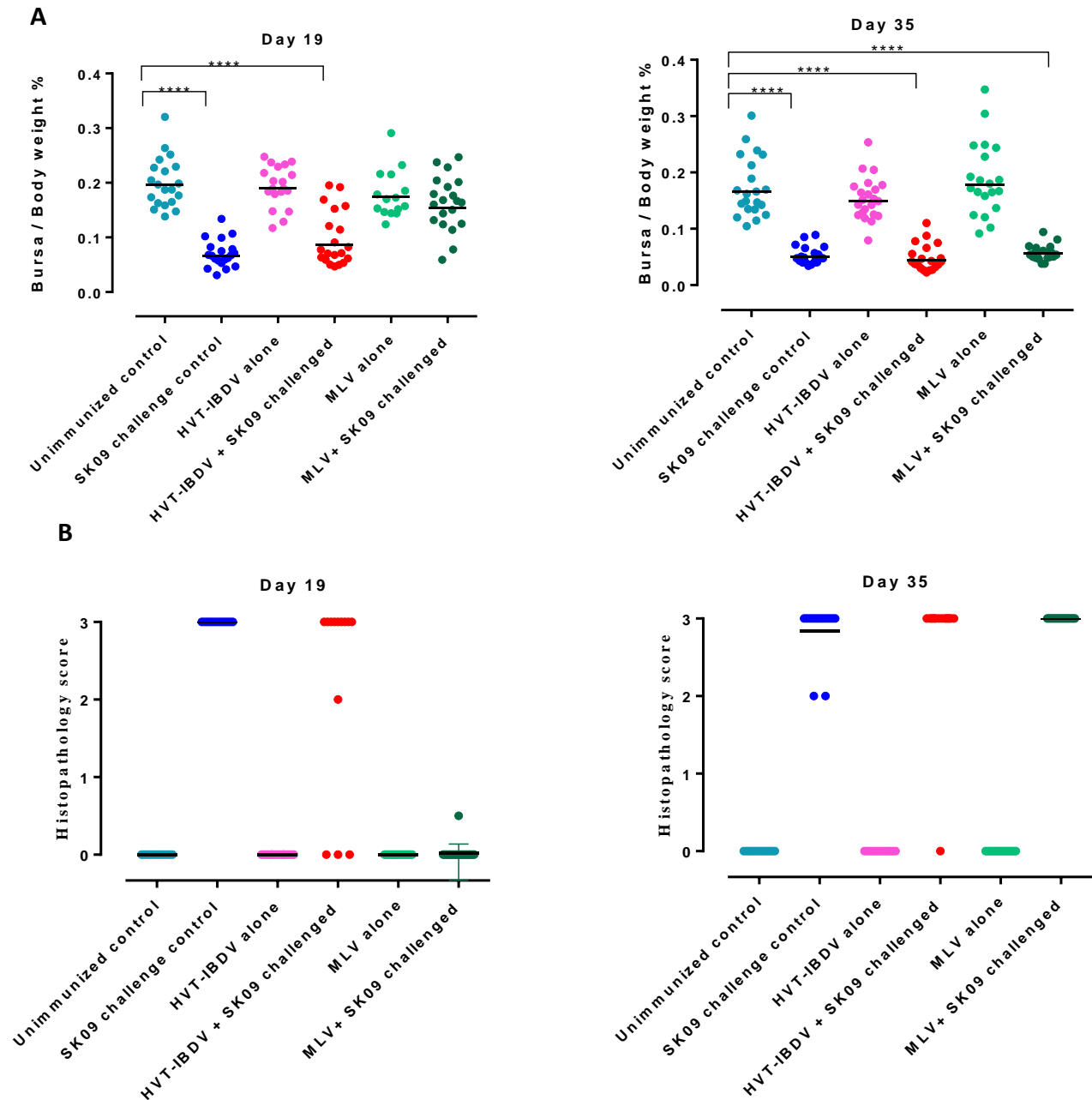


Figure 4-2: BBW and bursal histopathology of unimmunized (saline) or v-IBDV-SK09 challenged or HVT-IBDV vaccinated or HVT-IBDV + vIBDV-SK09 or MLV vaccinated or MLV + vIBDV-SK09 chickens. BBW (A) and histopathological scores (B). Sample were collected at 19 and 35 d of age. Each dot or sign represent individual value of BBW or clinical score, standard deviation and mean of 20 birds respectively. Asterisks and the horizontal brackets= significantly different groups ($P < 0.05$)

4.4.3 Quantitative real time PCR

Following, vIBDV-SK09 challenge (6 d of age), viral load in the BF of unchallenged (negative control and vaccine alone groups) and challenged groups was examined by qRT-PCR of bursal tissue samples collected at 9, 20 and 35 d of age (Figure 4- 3). We could not detect virus load in saline control (naïve) and vaccinated alone groups throughout our experiment, as evidenced by no Ct detection or Ct value around 39, which was similar to Ct value detection in NTC during qRT-PCR. For the relative quantification, we used Ct value 39 for VP2 gene in order to calculate Δ Ct value in saline control (naïve) and then calculated $\Delta\Delta$ Ct value to calculate fold viral loads in various group relative to saline control (naïve). At 9 f of age (3 d pi), we could not detect virus in MLV alone (Group 5), MLV + vIBDV-SK09 challenged (group 6) and HVT-IBDV vaccine alone (group 3) groups. Unvaccinated but vIBDV-SK09 challenged (Group 2) showed ~ 2.25 folds higher viral load. Surprisingly, we found a significantly high viral load (~ 5 744 folds) in HVT-IBDV vaccinated and vIBDV-SK09 challenged (Group 4), suggesting that HVT-IBDV vaccination facilitated vIBDV-SK09 replication. At 20 d of age, unvaccinated but vIBDV-SK09 challenged (Group 2) showed the highest viral load (~6 383 fold). Whereas, HVT-IBDV vaccinated and vIBDV-SK09 challenged (Group 4) and MLV + vIBDV-SK09 challenged (Group 6) groups showed ~ 732 and ~ 5.23 folds higher viral load, respectively. qRT-PCR data of 35 d of age bursal tissue samples showed about 3.11, 2.84 and 192 folds higher viral load in Group 2, Group 4 and Group 6, respectively. This data suggests that MLV vaccination delayed vIBDV-SK09 replication, as evidenced by significant viral load was not detected until 35 d of age.

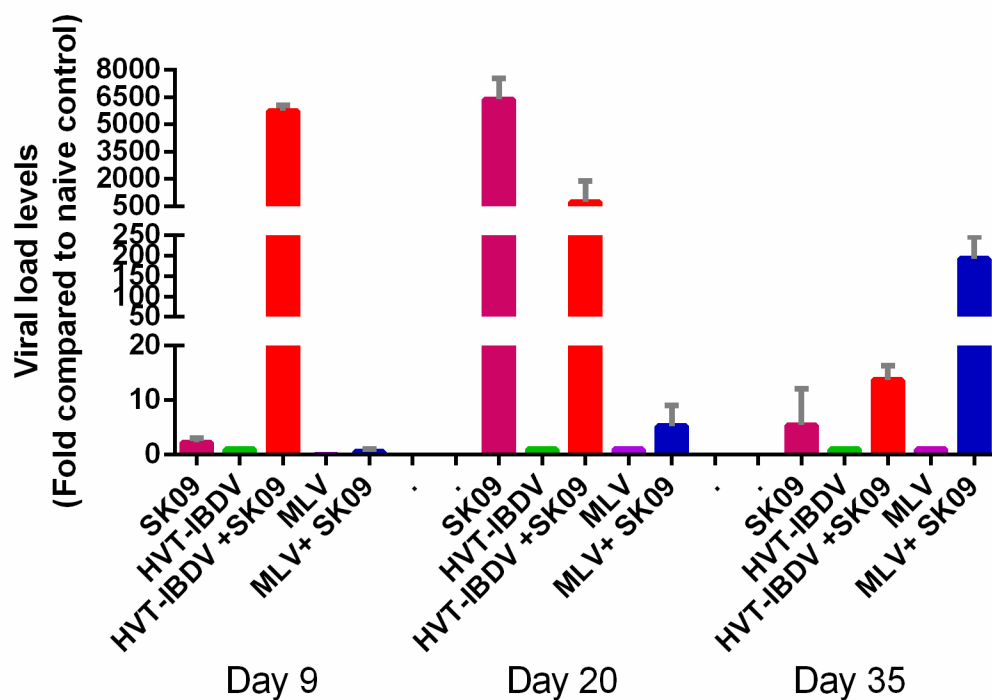


Figure 4-3: Relative quantification of IBDV load in BF of broiler chickens vaccinated with HVT-IBDV or MLV vaccine. Results are expressed as N-fold increase in the viral load compared to the saline control. Mean values were calculated from 3-4 bursa samples collected at indicated days and qRT-PCR was carried out in triplicates. Error bars indicate the standard deviations. MLV = modified live vaccine; HVT-IBDV = herpesvirus of turkey carrying IBDV VP2 gene; SK09 = vIBDV challenge virus.

4.4.4 Flow cytometry analysis

Flow cytometric analysis of splenic lymphocytes isolated from saline control (Group 1), HVT-IBDV alone (Group 3) and MLV alone (Group 5) at 8 d of age showed significant effects of vaccination on T-cells count (Figure 4A and Figure 4B).

(a) T lymphocytes (CD4 and CD8) count

Compared to saline, the MLV vaccinated group revealed an increase in the number of CD4⁺ and CD8⁺ T-cell counts. In contrast, the HVT-IBDV vaccinated group showed a significant decrease in the number of total CD4⁺ and CD8⁺ T-cells (Figure 4-4 A and Figure 4-4B).

(b) Cytotoxic CD8⁺ T-cell response

Given that cytotoxic CD8⁺ T-cells play crucial role against viral and infectious diseases (286, 287), we next examined the activation status of CD8⁺ T-cells. Cellular adhesion molecule, CD44, plays an important role in attachment, migration and T-cell activation. Upregulation of CD44 expression on T-cells is a well-established indicator of T-cell activation and thus the level of cell-surface CD44 expression is used as a phenotypic marker to distinguish naïve and activated T-cells in mice and human (286). Therefore, in order to evaluate the activation state of CD8⁺ T-cells in vaccinated groups, we compared CD44 expression on CD8⁺ lymphocytes of HVT-IBDV and MLV vaccinated chickens with the saline control. Flow cytometric analysis revealed significant upregulation of CD44 expression in MLV vaccinated chicken (Figure4- 4C, left panel). Interestingly, the HVT-IBDV vaccinated group showed down-regulation of CD44 expression on CD8⁺ lymphocytes, even lower than the basal expression level of saline group (Figure4-4C, right panel), suggesting immunosuppression in HVT-IBDV vaccinated chickens.

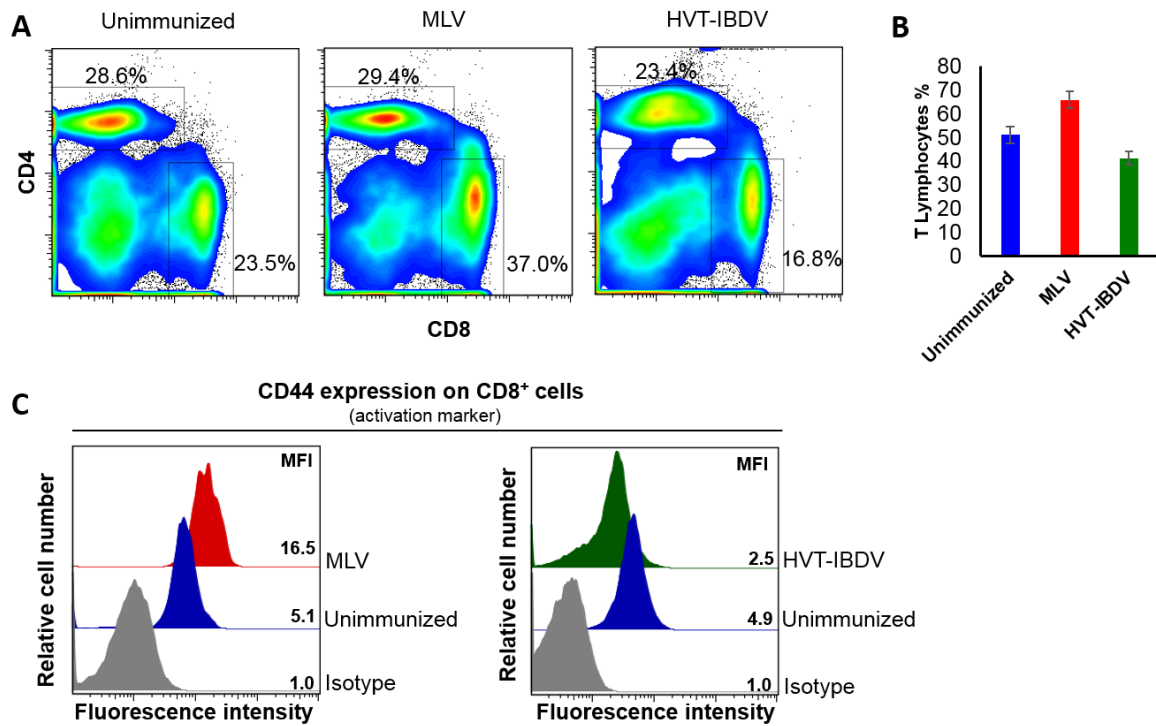


Figure 4-4: Flow cytometric analysis of splenic lymphocytes isolated from saline control, HVT-IBDV alone and MLV alone groups at 8 d of age. (A) Lymphocytes were gated based on forward and side scatter. T-cells were identified by CD3 staining. CD4⁺ T-cells and CD8⁺ T-cells were quantified using PE-labelled mouse anti-chicken CD4 and FITC-labelled mouse anti-chicken CD8 monoclonal antibodies. (B) Bar diagram indicate total number of CD4⁺ T-cells and CD8⁺ T-cells combined. (C) Histogram panel indicates the level of CD44 expression (an adhesion molecule and T-cell activation marker) on cytotoxic CD8⁺ T-cells of saline control, HVT-IBDV alone and MLV alone groups at 8 d of age. MFI = geometric mean fluorescence intensity.

4.4 Discussion

IBD is a disease of major economic importance (239) and its control is one of the most difficult challenges in poultry industry worldwide (156). As IBDV is resistant to many chemical disinfectants and environmental factors; once a poultry farm becomes contaminated with the virus, IBDV infection continue to occur and spread in subsequent flocks (288, 239). Therefore, vaccination against IBD constitutes the principal control measures of IBDV infection in chickens (289). Hyper-immunization of broiler breeders has been recognized as the most common strategy of control, where MAb may protect chickens up to 3 weeks of age (276). However, vIBDV can escape MAb acquired immunity (125, 122) and our recent study demonstrated that current broiler breeder vaccinations are not able to efficiently control the circulating vIBDV-SK-09 infection in chicks (276). When chicks become infected with IBDV during the critical stage (first 3 weeks of age), immunosuppression ensues, resulting in impaired vaccine response and increased susceptibility to secondary infections (250). Therefore, some poultry producers combine broiler vaccination using attenuated live IBDV or vectored vaccines (253). Compared to the attenuated live IBD vaccine, the HVT-IBDV vectored vaccine is considered safer, stable and effective against IBDV infections (277, 279, 280, 278, 281). Unlike previous studies (277, 279, 280, 278, 281), we primarily focussed on early age infection (6 d of age) with vIBDV-SK09 and compared the protective effects of two commercial broiler vaccines frequently used among Canadian broiler producers.

In field conditions, IBDV broiler vaccines are administered to broiler chicks obtained from hyper-immunized broiler breeders. Thus, the present study investigated the protective efficacy of HVT-IBDV and MLV vaccines against vIBDV-SK09 infection using chicks carrying MAb. Following *in ovo* HVT-IBDV vaccination in 18 d old embryo or subcutaneous MLV vaccination of day-old chicks, the development of antibodies was monitored using ProFLOK IBD plus ELISA kit. Serological analysis revealed that day-old chicks had high MAb titers and IBDV specific antibody titers declined over time as evidenced by ELISA titers at 19 d of age which declined to a barely detectable level by 35 d of age. However, ELISA titers of the serum samples collected from birds immunized with broiler vaccines (HVT-IBDV and MLV) at 19 d of age showed that despite high MAb, both HVT-IBDV and MLV vaccines successfully induced humoral immune response against IBDV. In general, the ability of the IBDV vaccines to break through relatively high MAb titers is crucial for the success of vaccination in field situations. But serological analysis at 35 d of

age revealed a significant decrease and antibody titers were at the lowest in both the HVT-IBD vaccine and MLV groups. In contrast, serological analysis in vaccinated + challenged groups (HVT-IBDV + vIBDV-SK09 and MLV + vIBDV-SK09) showed a significant increase in IBDV specific antibody titer at 35 d of age, suggesting a booster effect and seroconversion. Histopathological examination of bursal samples at 19 and 35 d of age did not show bursal lesions in either HVT-IBDV alone or MLV alone groups suggesting that the administration of these vaccines through the indicated routes are safe with no detrimental effects on BF. Studies reporting that IBD live vaccines cause significant bursal damage after vaccination used either the *in ovo* (278) or oral routes (277) of immunization. In this study, we used the subcutaneous route for MLV vaccination which did not cause bursal damage. Our finding is in agreement with previous studies that demonstrated no bursal damage following subcutaneous vaccination with IBD live vaccines in day old broiler chicks (290) and *in ovo* vaccination with HVT-IBDV (33).

In spite of a successful immunization with HVT-IBDV and MLV, vIBDV-SK09 was able to inflict severe bursal atrophy both in HVT-IBDV and MLV vaccinated birds, as evidenced by the histopathological examination of bursal samples and bursal weight measurement at 35 d of age. These data suggest that both vaccines failed to provide complete protection in chicks from an early age infection with vIBDV-SK09. Interestingly, histopathological scores and BBW data at 19 d of age showed severe bursal damage and low BBW values in the HVT-IBDV vaccinated group but not in the MLV group after the challenge with vIBDV-SK-09. These findings indicate that challenge virus, vIBDV-SK09, was able to inflict bursal damage and lymphoid depletion in the HVT-IBDV vaccinated group and further suggested that the MLV probably delayed viral pathogenesis. The delay in vIBDV pathogenesis in the MLV group could be the result of competition between the vaccine virus and the challenge virus. Previous studies have also reported competition between a mild and a pathogenic strain of IBDV, which suggested that such phenomenon could be either due to competition for host receptor sites or interference by cytokine(s) production (291). Alternatively, MLV induced innate immune response and T-cell responses could also play a role in restricting the challenge virus from damaging the BF (287). Our FACS analysis at 8 d post MLV vaccination revealed an increase in T lymphocytes (CD4 and CD8) as well as CD8⁺ T cell activation as evidenced by the upregulation of CD44 expression on CD8⁺ T-cells, which is in agreement with previous studies reporting peak T-cell responses against IBDV by 7 dpi (287, 292). Thus, MLV could delay vIBDV-SK09 pathogenesis through

competition and/or early immune mechanisms. Whatever may be the case, however, such interference phenomenon may have implications for vaccine mediated prevention of early age vIBDV infection in broilers.

We carried out qRT-PCR assay to detect viral load kinetics in bursal tissue at 9 (3 d pi), 20 (14 dpi) and 35 (29 dpi) d of age. We could not detect vaccine virus by qRT-PCR both in HVT-IBDV alone and MLV alone groups throughout our experiment. A previous study also reported the inability to detect vaccine virus in BF, which could be due to the localization of IBDV vaccine virus in the blood or other tissues not investigated here (272). After challenge, qRT-PCR analysis revealed low viral load at 9 and 20 d of age in the MLV + vIBDV-SK09 challenged group. However, the MLV + vIBDV-SK09 challenged group showed an increase in viral load later at 35 d of age. This result of delayed viral replication is in agreement with our histopathological scores that also showed bursal damage in the MLV + vIBDV-SK09 challenged group. Interestingly, 3 d pi with vIBDV-SK09 (challenge at 9 d of age), qRT-PCR assays revealed very high viral load in bursal tissues of the HVT-IBDV vaccinated group, which was significantly higher than the unvaccinated + vIBDV-SK09 challenged or MLV + vIBDV-SK09 challenged groups. It was surprising to find that 3 d pi following vIBDV-SK09 challenge, birds which were previously immunized with the protective HVT-IBDV vaccine revealed significantly high viral load when compared to the unprotected birds (unimmunized control).

FACS (at 8 d of age) of splenic lymphocytes after HVT-IBDV vaccination revealed that vaccination caused not only a significant decrease in total CD4 and CD8 T-cell numbers but surprisingly also down-regulated CD44 expression (adhesion molecule and activation marker) on cytotoxic CD8⁺ T-cells, suggesting vaccine induced immunosuppression. Our finding is in agreement with a previous report, which provided evidence that the HVT vaccine virus has the potential to immunosuppress broiler chickens during the initial 3-7 d of age by depleting leucocytes and lymphocytes count in vaccinated birds without affecting humoral response (283). It is noteworthy to mention that T-cells are important against IBDV infection and for limiting the disease severity (287) and T-cell suppression during an IBDV infection leads to an increase in viral replication and severe bursal damage (293). Thus, our present finding of enhanced vIBDV replication in the HVT-IBDV vaccinated group could be due to the HVT induced suppression of leukocytes and lymphocytes (283), leading to decreased immune pressure against an vIBDV

infection, thereby facilitating rapid early viral growth in the host, consequently leading to early bursal damage.

In conclusion, the MLV but not the HVT-IBDV vaccine was able to delay vIBDV-SK09 pathogenesis following early age infection. However, neither vaccines provided complete protection against early infection with vIBDV-SK09 as indicated by severe bursal damage by 35 d age. Our early-age challenge model of a vIBDV infection revealed a previously unrecognized aspect of the HVT-IBDV vaccine by showing that this vaccine may increase the vulnerability of birds to IBDV infection in the critical early phase of life. Overall, the present data has implications for vaccine design strategies that may help in optimizing an IBDV vaccination program in broilers.

PREFACE TO CHAPTER 5

Hyper-immunization of broiler breeder parents has long been practised as the main control strategy for IBDV in broiler progenies (294). Use of conventional IBD vaccination and proper bio-security measures were efficient in controlling IBD until the emergence of highly pathogenic and antigenic variant IBDV strains (133) (275). In the second and third chapters, we have demonstrated that current broiler breeder and broiler vaccines were not able to control the immunosuppressive effects of vIBDV circulating in the Canadian broiler chicken industry. Moreover, we have also demonstrated that vIBDV circulating in the Canadian broiler chicken industry were immunosuppressive. It has also demonstrated that vIBDVs in the Canadian broiler chicken industry are causing a significant economic loss to the poultry industry. Furthermore, it has also been demonstrated recently that an increased condemnation of broiler carcasses due to bacterial hepatitis at processing was associated with immunosuppression due to vIBDV. Thus, it is important to investigate the potential novel strategies of controlling the new vIBDV circulating across the country. The fifth chapter is demonstrating possibility of using vIBDV as a new vaccine candidate to control vIBDV infections in the Canadian broiler chicken industry.

CHAPTER 5 : IMMUNOPROTECTIVE EFFECTS OF VARIANT IBDV SK09 AS A VACCINE CANDIDATE AGAINST VARIANT INFECTIOUS BURSAL DISEASE VIRUSES IN THE CANADIAN BROILER CHICKEN INDUSTRY

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5.1 Abstract

vIBDV strains and their association with significant economic losses are well-known in the Canadian broiler chicken industry. Current broiler breeder vaccination programs practiced in Canada are not very effective in controlling vIBDV. The transfer of MAb to progenies by hyperimmunization of broiler breeder parents has been recognized as the main strategy for controlling IBDV. The objective of this study was to test vIBDV-SK09 as a potential vaccine candidate in the broiler breeder vaccination program. Five groups of broiler breeders were vaccinated at 13 weeks of age with live vIBDVs; SK09 (NC171 “like”), SK10 (05SA8 “like”), SK11 (Delware-E “like”), SK12 (586 “like”), SK13 (Prezotto-BR “like”). One group of breeders served as unvaccinated control. Another group of commercial broilers were compared as positive control. Broiler progenies of vaccinated breeders and commercial broilers were challenged with vIBDV-SK09 at 6 d of age. Antibody titers against IBDV, BBW, viral load in the BF and bursal histopathology of broilers were studied to evaluate efficacy of vIBDV-SK09 as a vaccine candidate. Seroconversion against IBDV, bursal damage and viral load of BF were least in the group of broiler originated from parents which were vaccinated with vIBDV-SK09 compared to commercial broilers originated from conventional broiler breeder vaccination program practiced in Canada. Although, we have not vaccinated broiler breeders multiple times with live followed by inactivated vaccines as practiced in the commercial broiler breeder industry, yet the pilot study with a single exposure of broiler breeders to live vIBDVs currently circulating in Canada has demonstrated that the antigenically relevant vIBDV -SK09 could be a potential vaccine candidate against IBDV infection in Canadian chicken industry.

5.2 Introduction

IBDV, a member of *Birnaviridae*, is a double stranded RNA virus which is a leading cause of immunosuppression in chickens. IBD was first reported in Gumboro, Delaware, USA in 1962 (201) . Currently, IBDV has a worldwide distribution affecting broiler industry with great economic significance (250). The main target cells of the virus are actively dividing immature B cells in BF (BF)(76). The pathogenic serotype I can be classified into 3 major strains based on antigenicity and pathogenicity; classical or standard IBDV, vIBDV and vv IBDV (24). IBDV in broilers has been controlled by a vaccination strategy to maximize MAb against IBDV by hyper-

immunization of broiler breeder parents. A common strategy is to administer a series of live attenuated vaccines followed by an inactivated vaccine “booster” that results in high levels of MAb in the progeny (156).

Currently, variant strains have been recognized as the main IBDV strain circulating in the USA and Canada (130, 123, 129). These antigenically variant strains can escape immunity elicited by the conventional vaccine strains and cause rapid, and permanent bursal atrophy, and lymphoid cell depletion (110) (111, 112) (113). In the past, there has been a significant rise in IBDV infection in Canadian broiler chicken farms associated with production losses, clinical signs associated with respiratory and enteric diseases and vaccine failures (129). Several studies reported that the majority of the IBDV strains circulating in Canada are variants (130, 129). Variant IBDV infection is classified as sub-clinical form of IBD, in which “problem flocks” are presented without any visible clinical signs until the flock succumbs to secondary infections, poor weight gain, reduced feed conversion and poor performance (114). We have identified five main vIBDV strains from the poultry industry in Canada that are responsible for immunosuppression and associated production losses (295, 276); SK09 (NC171 “like”), SK10 (05SA8 “like”), SK11 (Delaware-E “like”), SK12 (586 “like”), SK13 (Prezotto-BR “like”). Commercially available current broiler breeder or and broiler vaccines are not able to control these vIBDVs (manuscript submitted for the publication). This pilot study was designed to test the suitability of vIBDV-SK09 as a broiler-breeder vaccine candidate.

5.3 Materials and methods

5.3.1 Vaccination of broiler breeders with vIBDVs as vaccine antigens

The objective of this study was to evaluate the efficacy of vIBDVs as vaccine candidates in broiler breeders. Variant IBDVs; SK09, SK10, SK11, SK12 and SK13 that have been isolated in the Canadian broiler chicken industry were used as vaccine antigens (Figure 5-1)

Day-old broiler breeders were obtained from Aviagen Inc., (Huntsville, AL) and housed them at the ACU, Western College of Veterinary Medicine, University of Saskatchewan. Feeding and lighting programs were conducted according to the Aviagen, Inc. guidelines for broiler breeders. Six groups of broiler breeders, each group containing 20 females and 4 males were raised in isolation rooms. At 13 weeks of age, groups were vaccinated with SK09, SK10, SK11, SK12 and SK13 or saline prepared from BF of SPF birds. Each bird received 3×10^3 EID₅₀ of vIBDVs by

the oral route. Serum samples were collected from all groups (n=20, each group) at 3, 7 and 20 weeks post-vaccination to determine antibodies against IBDV. Eggs from broiler breeders were collected at 21 week post-vaccination and incubated to produce their respective progenies.

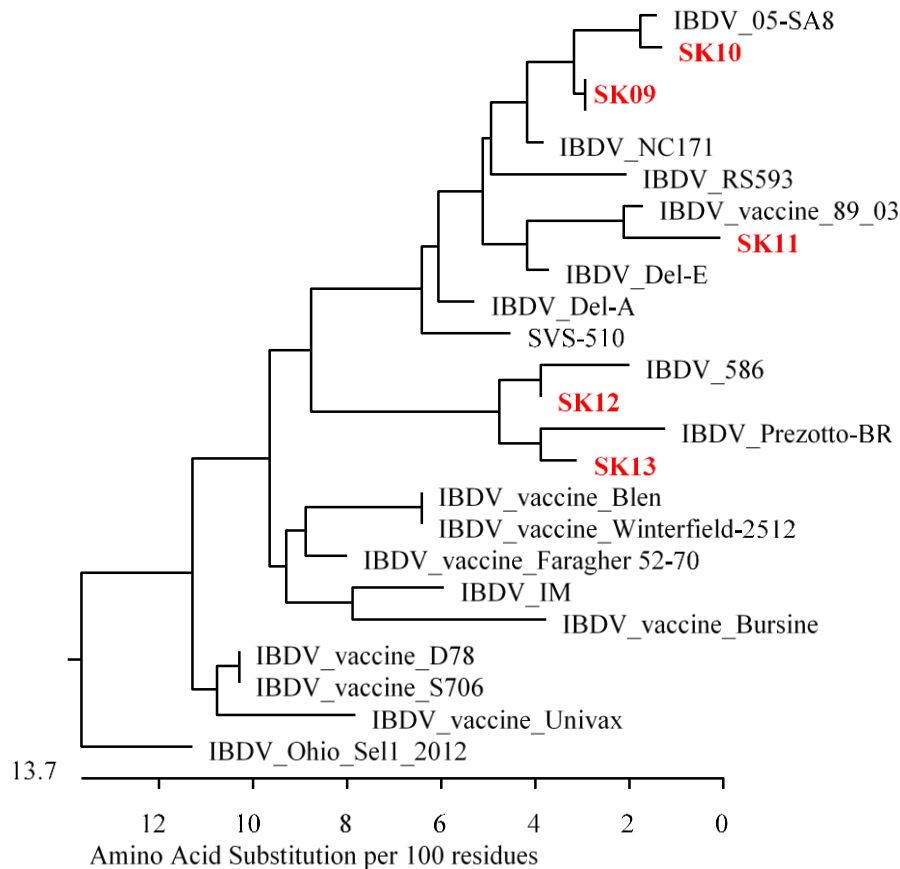


Figure 5-1: Phylogenetic analysis of vIBDV circulating in Canada

5.3.2 Challenge of broiler progenies with vIBDV-SK09

Groups of day-old broiler chicks (n=40) from their parents vaccinated with SK09, SK10, SK11, SK12 and SK13 or saline were collected and placed at the ACU. Another group of day old broilers (n=40) were obtained from a commercial hatchery (Prairie Pride Chick Sales Ltd) Broiler breeder parents of these broilers were vaccinated against IBDV at 14 d of age with Bursine 2 (Zoetis, Kirkland, Quebec), 21 d of age with Bursimune (Ceva Animal Health, Cambridge, ON), 8 weeks of age with Bursa Blen M (Merial, Gainesville, GA), 10 weeks of age with Matimavac and 18 weeks of age with Maximune Avi-Pro 432 ND-IB2-BD3 REO (Lohmann Animal Health International, Winslow, ME). Feed and water were provided *ad libitum* to the broilers. Sera was collected from 10 birds per group at hatch. All groups (n=40) of broilers were orally inoculated with 3×10^3 EID₅₀ of vIBDV-SK09 at 6 d of age.

5.3.3 Broiler breeder vIBDV vaccine and vIBDV challenge preparation of broilers

Vaccine (SK09, SK10, SK11, SK12 and SK13) and challenge viruses (vIBDV-SK09) were prepared following standard procedures (136). Briefly, pooled bursal tissue samples collected from vIBDV infected birds were homogenized in PBS to make 40% (w/v) suspensions. The suspensions were centrifuged 3,000 rpm for 10 min and filtered through 45 nm pore size filter. The filtrate was orally administered to 18-d-old SPF chickens (Sunrise Farms, Inc. Catskill, NY). BF were collected three days following vIBDV infection and vIBDV was titrated in SPF embryos as described previously (136). In brief, 10-d-old embryonated SPF chicken eggs were inoculated with 0.1 ml of filtered (45 nm pore size) bursa homogenate diluted 10-fold in PBS solution (pH 7, containing 10 µg/ml gentamicin sulfate (Gibco, Invitrogen Corp, Carlsbad, CA) via the CAM. The EID₅₀ was determined using the Reed and Munch method (136).

5.3.4 ELISA, BBW and histopathology of BF

At 19 and 35 d of age, 20 broilers from each group were bled for sera collection before euthanizing to obtain BBW and histopathology of BF. Sera samples were tested for antibodies against IBDV using a commercial ELISA kit according to manufacturer's instructions (IDEXX-XR, Westbrook, ME). BBW of each bird was determined at necropsy and calculated as bursal weight (g)/body weight (g) x 100 (%). Sections of BF were processed for histopathology by fixing in 10% neutral-buffered-formalin. Fixed tissues were embedded in paraffin and sectioned at 5 µm and stained with

H&E. Sections of BF were graded on the basis of lymphoid atrophy as follows: 0 = no visible lesions; 1 = mild, focal to multifocal lymphoid atrophy; 2 = moderate, multifocal lymphoid atrophy; and 3 = severe, diffuse lymphoid atrophy (Figure 5-2).

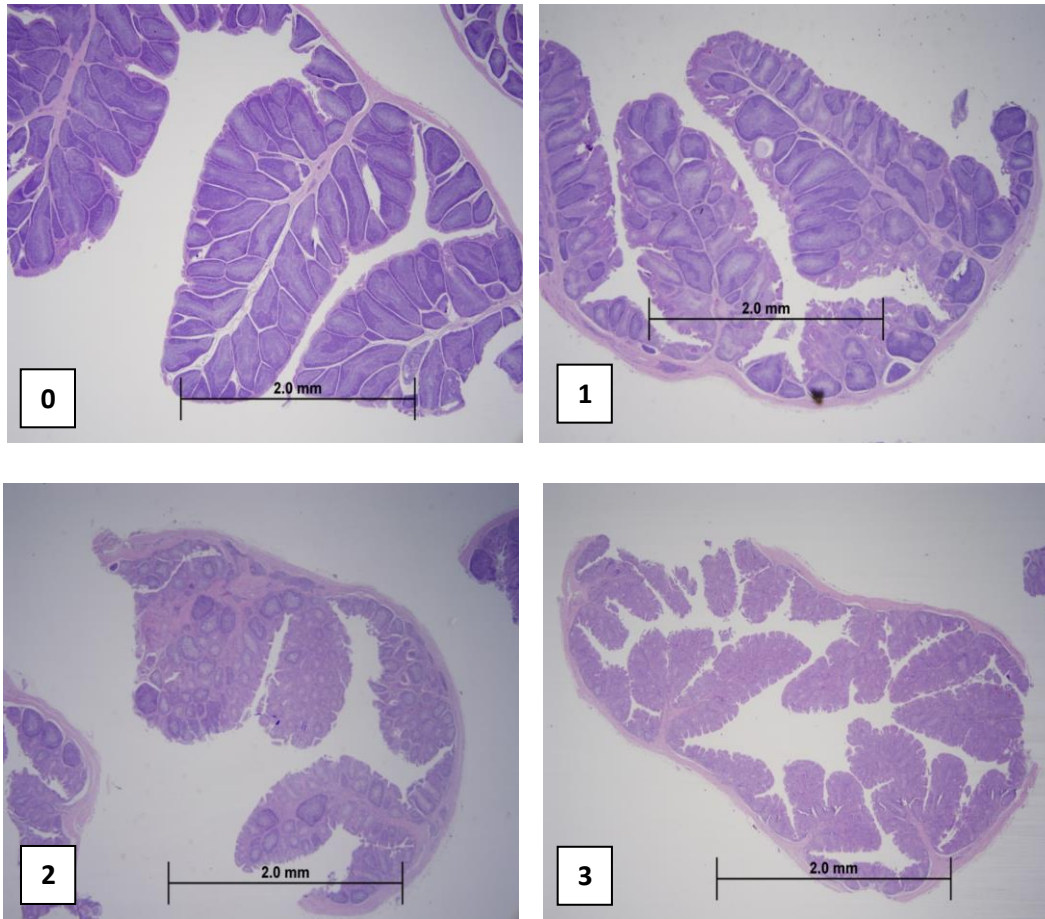


Figure 5-2: Histopathological scoring of BF following exposure of birds to vIBDV. BF was graded on the basis of lymphoid atrophy as follows; 0 = no visible lesions; 1 = mild, focal to multifocal lymphoid atrophy; 2 = moderate, multifocal lymphoid atrophy; and 3 = severe, diffuse lymphoid atrophy.

5.3.5 RNA isolation and reverse transcriptase-polymerase chain reaction

Following vIBDV-SK09 challenge, samples of BF (n=3) were collected at 19 d of age. About 5 mg of tissues were used for extracting total RNA using RNeasy mini kit (Qiagen, Mississauga, ON) following the manufacturers protocol. Total RNA from each sample was

dissolved in 50 µl of RNase-free water. RNA quality was determined by agarose gel electrophoresis. RNA was quantified spectrophotometrically using NanoDrop 2000 (Thermo Fisher Scientific). First-strand cDNA was synthesized with 1 µg of total RNA, Moloney Murine Leukemia Virus Reverse Transcriptase and primers (oligo-dT and random hexamer mixed) using QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON) in 20 µl volume, according to manufacturer's protocol. All cDNA samples were stored in -80 °C until further use.

5.3.6 Real-time PCR analysis for the quantification of IBDV viral load in bursal tissue

Viral load quantification in bursal tissue was determined using real-time PCR (Mx3000P qPCR system, Agilent Technologies) and TaqMan probes (285). The primers to amplify a 74 bp fragment of IBDV VP2 gene were forward primer 5'-GGACACAGGGTCAGGGTCAAT-3' (VP2-F) and reverse primer 5'-GCAGTGTGTAGTGAGCACCCA-3' (VP2-R). The TaqMan probe used to identify 74 bp VP2 fragment was 5'-TCTTTTCCCTGGATTCCCTGGCTCA-3', which was labelled with FAM (reporter dye) and ZEN/IBFQ (double quencher) at 5' and 3', respectively. To quantify relative abundance, chicken 18S rRNA was used as housekeeping gene to normalize the quantity of cDNA in PCR reactions. TaqMan primers used to amplify a 186 bp fragment of chicken 18S rRNA were as follows, forward primer 5'-CGGCTACCACATCCAAGGAA-3' (18S-F) and reverse primer 5'-GCTGGAATTACCGCGGCT-3' (18S-R). The TaqMan probe used to detect 18S rRNA target was 5'-TGCTGGCACCAGACTTGCCCTC-3', which was labelled with HEX reporter dye at 5' and ZEN/IBFQ double quencher at 3'. The IBDV VP2 and 18S RNA amplifications were performed in the same tube (20 µl total volume) using Prime Time-Gene Expression Master Mix (IDT) and 2 µl of cDNA template. The PCR amplification conditions were, initial melting at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 s at 60 °C. Serially diluted cDNA samples were used to determine PCR efficiency. Real-time PCR data were expressed as Ct value, that is, the number of cycle of PCR at which the fluorescence emitted by the reporter dye crosses a threshold fluorescence value as determined by the software of PCR machine. The lower Ct value suggests that fluorescence intensity of reporter dye passed threshold early indicating higher the initial amount of DNA of interest. For real-time PCR, samples were used in triplicate, maintaining NTC, IBDV negative and IBDV positive samples in each experiment. IBDV viral loads in bursal tissues

were determined by comparative Ct method ($\Delta\Delta\text{Ct}$ method) by first normalizing data for differences in the amount of RNA among samples using 18S rRNA Ct value of each sample. Ct value of VP2 NTC and naïve samples were either not detected or were around 39. Therefore, naïve control Ct value was set 39 in calculation. Following formula was used to estimate comparative viral load in bursal tissues:

$$\Delta\text{Ct (test sample)} = \text{Ct value of VP2 (test sample)} - \text{Ct value of 18S sRNA (test sample)}$$

$$\Delta\text{Ct (naïve control)} = \text{Ct value of VP2 (naïve control)} - \text{Ct value of 18S sRNA (naïve control)}$$

$$\Delta\Delta\text{Ct} = \Delta\text{Ct (test sample)} - \Delta\text{Ct (naïve control)}$$

$$\text{Fold} = 2^{-\Delta\Delta\text{Ct}}$$

5.3.7 Statistical analysis

The BBW, histopathological score and antibody titer against IBDV were analysed using Wilcoxon Rank Sum Test (to compare 2 groups) or Kruskal–Wallis One-way ANOVA (to compare more than 2 groups). Survival and other data were analyzed with the use of Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA) and Statistix7 (Analytical Software, Tallahassee, FL) with a significance level of $P < 0.05$.

5.4 Results

5.4.1 Vaccination of broiler breeders with variant IBDVs as vaccine candidates

The geometric mean titers (GM) against SK09 were 11 403, 14 717 and 11 660 at 3, 7 and 20 weeks post-vaccination respectively. Antibody titers (GM) against SK10 were 10 975, 11 245 and 12,224 at 3, 7 and 20 week PV respectively. Antibody titers (GM) against SK11 were 7 114, 3 696 and 3 309 at 3, 7 and 20 weeks post-vaccination respectively. Antibody titers (GM) against SK12 were 8 599, 7 765 and 9 177 at 3, 7 and 20 weeks post-vaccination respectively. Antibody titers (GM) against SK13 were 8 516, 8864 and 10205 at 3, 7 and 20 weeks post-vaccination respectively (Figure 5-3).

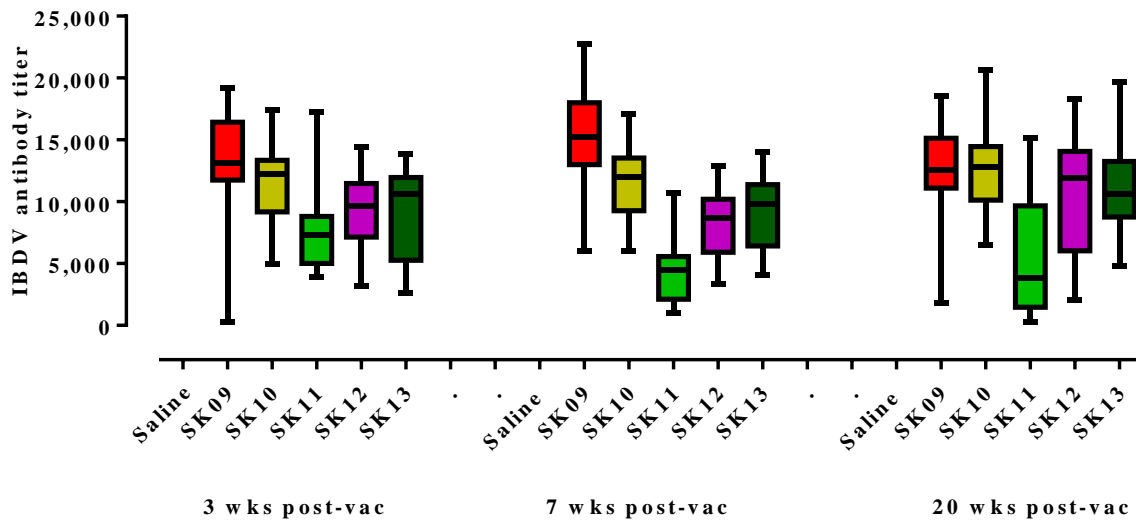


Figure 5-3: IBDV antibody titer of vaccinated breeders at 3, 7 and 20 weeks post-vaccination. The group that did not receive vIBDV did not have antibodies against IBDV and remaining groups administered with vIBDV seroconverted.

5.4.2 Challenge of broiler progenies with SK09

MAB titers (GM) against SK09, SK10, SK11, SK12, SK13 and commercial IBDV vaccines at hatch were 4 204, 1 346, 764, 1 882 1 900 and 4 408 respectively. Antibody titer against IBDV at hatch in the group originated from their parents not vaccinated with vIBDV was 4 (Figure 5-4).

At 19 d of age, antibody titers (GM) against IBDV in groups originated from their parents vaccinated with SK09, SK10, SK11, SK12, SK13 and commercial IBD vaccines were 155, 147, 354, 354, 346 and 2 542 respectively. At 35 d of age, antibody titers (GM) against IBDV in groups originated from their parents vaccinated with SK09, SK10, SK11, SK12, SK13 and commercial IBD vaccines were 219, 873, 1 601, 822, 1,308 and 4 911 respectively (Figure 5- 5). The broiler progenies that were challenged with vIBDV-SK09 at 6 d of age and originated from parents not vaccinated with vIBDV had GM antibody titers of 5 003 and 10 319 at 19 and 35 d of age respectively. Antibody titer in the broiler progeny originated from parents not vaccinated with vIBDV had a significantly higher antibodies against IBDV compared to broilers originated from parents vaccinated with vIBDV or commercial IBD vaccines ($P < 0.0001$). The broiler progenies

originated from parents vaccinated with SK09 had the lowest antibody titer (GM) against IBDV at 35 d of age ($219\pm$) (Figure 5-5). The group originating from parents vaccinated with the commercial IBDV vaccine had significantly higher antibody titers at 19 d ($P=0.003$) and 35 d of age compared to the group which originated from parents vaccinated with SK09 ($P=0.001$).

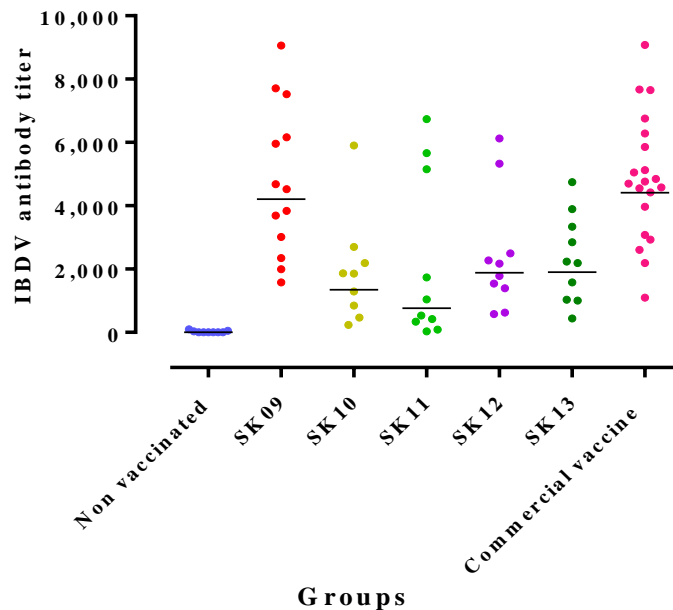


Figure 5-4: Maternal antibody titer against IBDV of broilers at the time of hatch. Broilers from their parents not vaccinated with vIBDV did not have antibodies against IBDV and remaining groups from their parents administered with vIBDVs had antibody against IBDV.

At 19 d of age, BBW of broilers originated from their parents vaccinated with vIBDV-SK09, SK10, SK11, SK12, SK13, commercial IBD vaccine and saline were 0.16, 0.17, 0.06, 0.13, 0.10, 0.06 and 0.16, respectively. BBW was lowest in the group of broilers from parents with no IBDV vaccination but challenged with SK09. BBWs were higher in the groups of broilers from their parents vaccinated with SK09, SK10 or birds that were not challenged with SK09. BBW was higher in the group of broilers from their parents vaccinated with SK09 compared to broilers originated from their parents vaccinated with commercial IBDV vaccine ($P=0.1$).

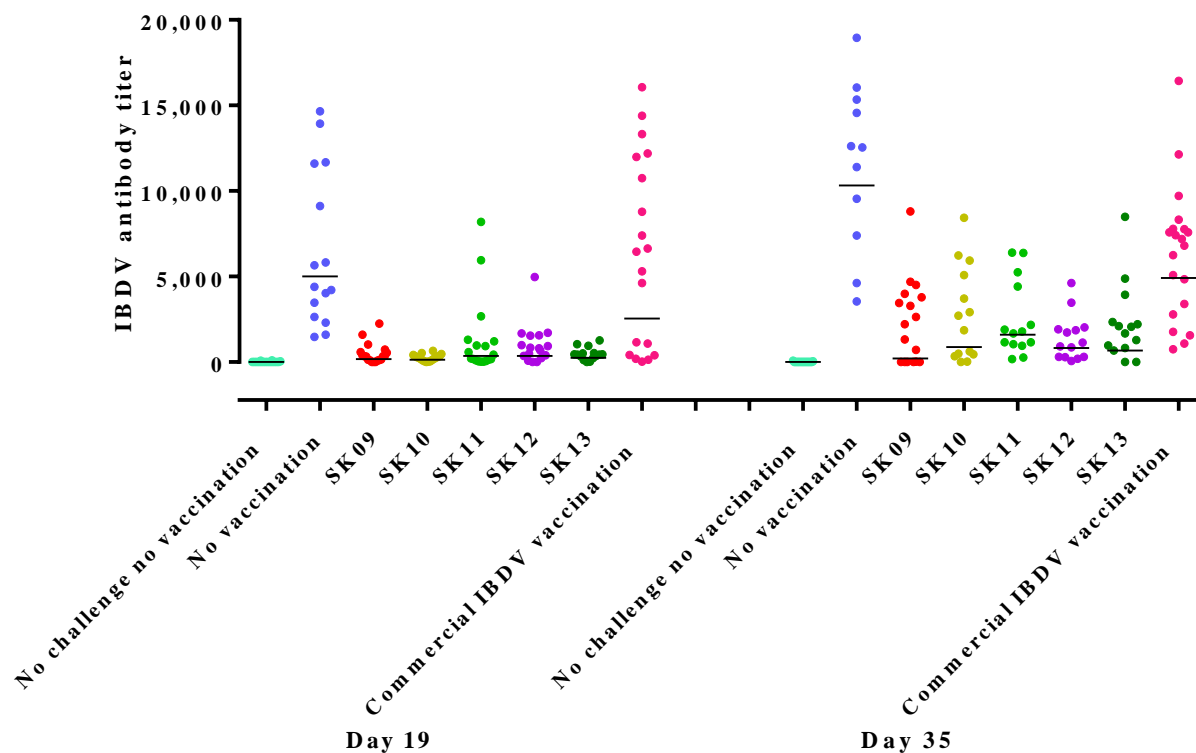


Figure 5-5: IBDV titers (GM) following vIBDV-SK09 challenge in groups of broilers from their parents vaccinated with vIBDV; SK09, SK10, SK11, SK12, SK13 or commercial broiler IBDV vaccine. All the groups were challenged with SK09 at 6 d of age except one group of broilers from their parents not vaccinated against IBDV (No challenge no vaccination). The highest antibody titer against IBDV was noted in the group of broilers from their parents not vaccinated against IBDV while the lowest antibody titer against IBDV was noted in the group of broilers from their parents vaccinated with SK09. IBDV titer of the group of broilers from their parents vaccinated with commercial IBDV vaccine was higher than broilers of their parents vaccinated with SK09.

At 35 d of age, BBW of broilers originated from parents vaccinated with SK09, SK10, SK11, SK12, SK13, commercial vaccine and saline were 0.08, 0.06, 0.05, 0.06, 0.04, 0.05 and 0.12, respectively. The BBW was highest in 35 d of age broilers in the group not challenged with SK09. The BBW was second highest in the group of broilers that originated from their parents vaccinated with SK09. The BBW was lowest at 35 d old broilers in the group challenged with SK09 and originated from parents were not vaccinated with vIBDV ($P>0.05$). At 35 d of age, BBWs were significantly lower in groups of broilers originated from their parents vaccinated with SK11, SK12 and SK13 compared to the group not challenged with SK09 ($P >0.05$). Broilers originated from their parents vaccinated with a commercial IBDV vaccine had lower BBW compared to broilers originated from their parents vaccinated with SK09 ($P =0.23$ (Figure 5-6).

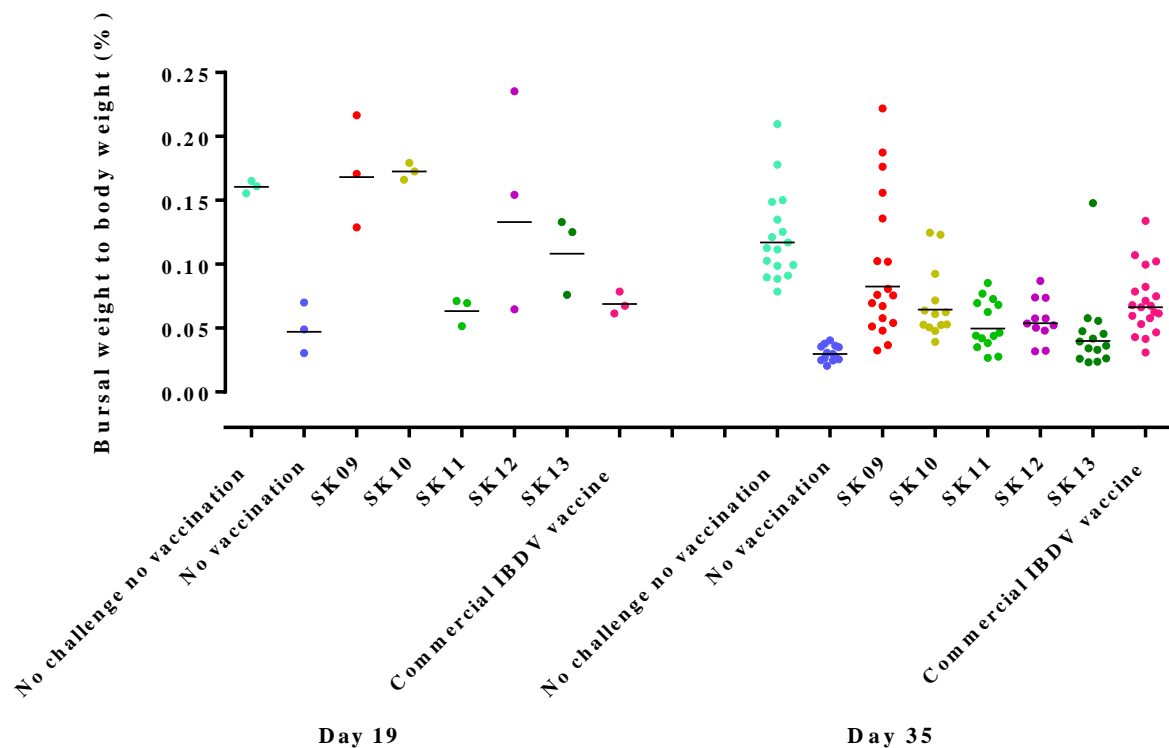


Figure 5-6: Bursal weight to body weight ratio (BBW) following vIBDV-SK09 challenge in groups of broilers from their parents vaccinated with vIBDVs; SK09, SK10, SK11, SK12 , SK13 or commercial broiler IBDV vaccine. All the groups were challenged with ISK09 at 6 d of age except one group of broilers from their parents not vaccinated against IBDV (No challenge no vaccination). The highest BBW was noted in the group of broilers from their parents not challenge with IBDV while the lowest BBW was noted in the group of broilers from their parents not vaccinated against IBDV. BBW was highest in the group of broilers from their parents vaccinated with SK09 among all vaccinated groups at 35 d of age.

Bursal atrophy was not noted at 35 d of age histologically in the group not challenged with SK09. A severe bursal atrophy was noted at 35 d of age in the group challenged with SK09 and originated from parents not vaccinated with vIBDV. Bursal atrophy was lowest in the group of broilers from their parents vaccinated with SK09 compared to groups of broilers originated from their parents vaccinated against vIBDVs including group of broilers from their parents vaccinated with commercial IBDV vaccine (Figure 5-7).

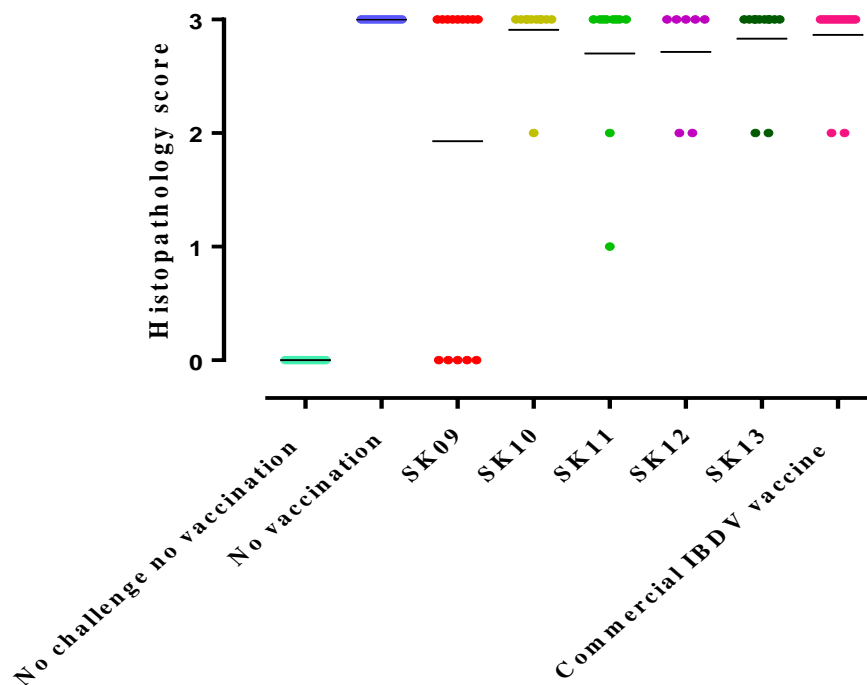


Figure 5-7: Histopathological score of broilers at 35 d of age following vIBDV-SK09 challenge in groups of broilers from their parents vaccinated with vIBDV; SK09, SK10, SK11, SK12, SK13 or commercial broiler IBDV vaccine. All the groups were challenged with SK09 at 6 d of age except one group of broilers from their parents not vaccinated against IBDV (No challenge no vaccination). The highest bursal damage was noted in the group of broilers from their parents not vaccinated against IBDV. The lowest bursal damage score was noted in the group of broilers from their parents vaccinated against SK09.

IBDV was detected in the BF in groups of broilers from their parents vaccinated with SK11, SK12, SK13, IBDV commercial vaccine or group of broilers from their parents not vaccinated against IBDV. IBDV was not detected in the BF of groups of broilers vaccinated against SK09 and SK10 (Figure 5-8).

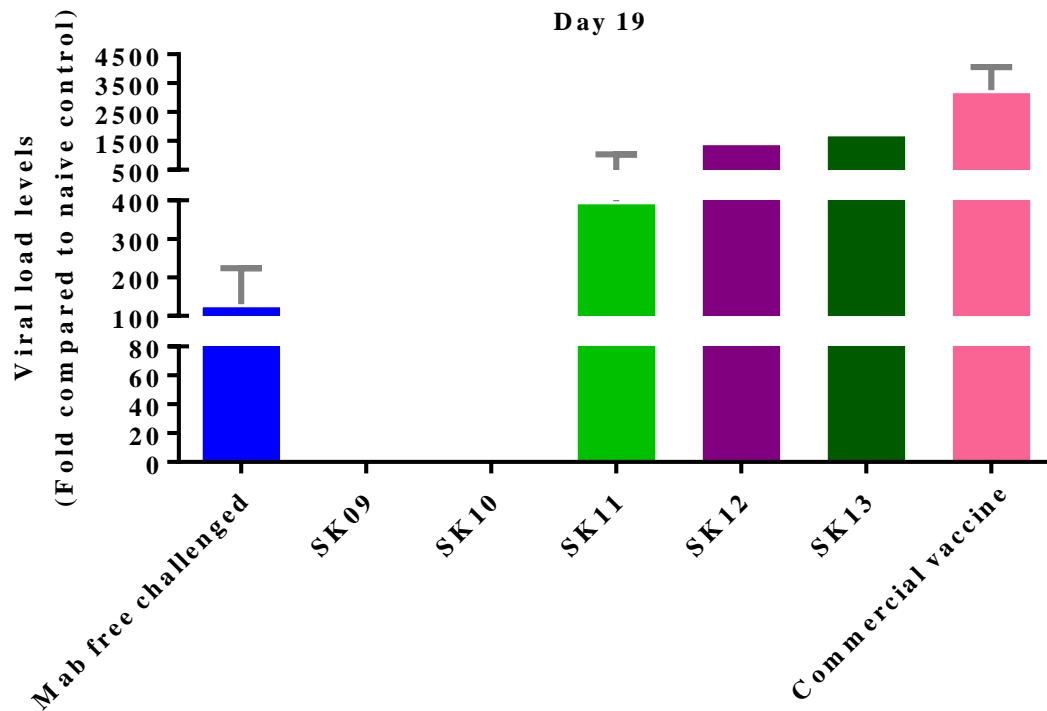


Figure 5-8: IBDV load in the BF at 19 d of age. Groups of broilers from their parents vaccinated with SK09, SK10, SK11, SK12, SK13 or commercial broiler IBDV vaccine following vIBDV-SK09 challenge at 6 d of age. No IBDV was detected in the BF groups of broilers from their parents vaccinated with SK09 or SK10

5.5 Discussion

IBDV infection of broilers has been controlled by vaccination of broiler breeders to maximize MAb against IBDV in broiler chicks. This has been achieved by hyper-immunization of broiler breeder parents to live and inactivated IBDVs repeatedly. A common vaccination strategy in broiler breeders is to administer a series of live attenuated vaccines followed by an inactivated vaccine “booster” that results in high levels of MAb in the progeny (156). Sometimes, IBDV control measures also combine a broiler vaccination program using attenuated IBDVs (253). Recent epidemiological studies conducted in the Canadian broiler chicken industry have confirmed that most of the variant IBDV strains are antigenically divergent and that can escape the immunity induced by conventional vaccine strains (130, 129). Moreover, we have demonstrated that current vaccination programs against IBDV are not very effective against vIBDV strains circulating in the Canadian broiler chicken industry (276). We have also demonstrated that Canadian vIBDV strains are immunosuppressive and cause significant economic losses to the poultry industry in Canada (276). Thus it emphasizes the urgent need of antigenically appropriate IBDV strains as vaccine candidates to control vIBDV in the Canadian broiler chicken industry. This study was design to examine the efficacy of circulating vIBDVs as vaccine candidates against most prevalent vIBDV SK09. Immunoprotective effects of vIBDV vaccines in broiler breeders were determined by evaluating antibody titer against IBDV, BBW, bursal damage and virus load in the BF of the broiler progeny following the challenge with vIBDV SK09. Broiler progenies originated from the parents vaccinated with SK09 had the lowest antibody titer against IBDV, moderate bursal atrophy, higher BBW and no detectable IBDV in the BF at 19 days of age. Moreover, no IBDV were detected in the BF of broilers from their parents vaccinated with SK10. This observations demonstrates that antigenically closely related IBDV can be controlled by SK09 as a vaccine candidate. SK09 and SK10 strains are closely related compared to SK11, SK12 and SK13 strains. Since broiler breeder parents were vaccinated only once using live IBDV in this study, the serum antibody titer against IBDV following vaccination were comparatively lower than the average antibody titer that we see in the field.

In summary, this study demonstrated strong immunoprotection in broilers against SK09 when their parents were vaccinated with SK09. Of note, antigenically related strains of SK09 such as SK10 was able to control SK09 challenge. This cross protection is likely associated with sequence similarity of SK09 to SK10 (97.5 %). Overall, results suggest that circulating vIBDVs

could be potential vaccine candidates. However, further studies are needed using other vIBDV strains as the challenge virus, besides comparing single and multiple vaccination regimens.

CHAPTER 6 : DISCUSSION AND CONCLUSIONS

IBD is a major concern to the poultry industry in Canada hence it is associated with significant production losses due to sub-clinical infections and secondary diseases. It has been recognized as an important infectious agent causing high mortality in acute infection and severe immunosuppression in sub-clinical infection. The high mutation rate of IBDV causes the emergence of antigenically variant strains which can escape conventional IBDV vaccine-induced immunity. In the USA and Canada, the sub-clinical infection caused by vIBDV have been recognized as the major underlying cause of immunosuppression which increases the susceptibility of chickens to many opportunistic pathogens, causing considerable economic losses to farmers. Therefore, it is essential to evaluate and understand the efficacy of current control strategies and implement new control measures if needed to prevent and control emerging vIBDV infections in the Canadian broiler chicken industry. The goals of this research project were to characterize immunosuppressive effects of field isolates of vIBDVs, evaluating the current control strategies against IBDV in broiler breeder parents and broiler chicken industries in Canada, as well as identifying a potential vaccine candidate IBDV strain(s) against immunosuppressive variant strains of IBDV circulating in Canada.

We undertook an extensive study on the most prevalent strain of IBDV (vIBDV-SK09) in the Canadian broiler chicken industry. We have demonstrated that vIBDV-SK09 was immunosuppressive in young broiler chickens, and are able escape from the MAb acquired by chicks via current broiler breeder parent vaccination programs. Further, we showed that current broiler vaccines were also not able to efficiently control vIBDV-SK09.

It was shown that despite the presence of MAb derived from hyperimmunized broiler breeder parents, vIBDV-SK09 was able to infect the BF and cause a significant bursal atrophy. We found low BBW, severe bursal atrophy and high antibody titers against IBDV following experimental infections in commercial broiler chickens with vIBDV-SK09, which is similar to field observations associated with immunosuppression suspected “problem flocks” mentioned in field studies conducted in the USA and Canada (123, 129). These flocks were typically associated

with conditions such as air sacculitis, tracheitis, pneumonia, septicemia, inclusion body hepatitis and coccidiosis. In order to demonstrate the clinical immunosuppression of vIBDV infection, we have exposed commercial broiler chickens to vIBDV-SK09 prior to challenge with an avian pathogenic *E. coli* strain (317). Immunosuppressed broiler chickens were more susceptible to *E. coli* challenge at a significant level compared to the control group not exposed to vIBDV-SK09. This observation further explains that infection of broilers with vIBDV-SK09, even in the presence of high titers of MAb to IBDV, resulted in a significant increase in their susceptibility to acute *E. coli* infection and caused septicemia and death. Furthermore, higher *E. coli* counts were seen in air sacs of birds challenged with vIBDV-SK09 and this is likely associated with that immunosuppressed birds had reduced ability to clear bacteria from the body and prevent septicemia. These data explain why we see disease problems and complications such as high mortality, chronic infections and high condemnations in broiler chickens raised in antibiotic-free farming, if those farms are infected with vIBDV. This will be very valuable information to poultry farmers that they need better and effective control measures against vIBDV infection in farms, prior to venturing and investing on antibiotic-free chicken farming.

In addition to hyper-immunization of broiler breeders, broiler vaccination is also important in controlling vIBDV in the broiler chicken industry, as such vaccination may induce both humoral and cellular immunity to IBDV (270). In newly hatch chickens, MAb specific to IBDV exhibits a curvilinear decline with age, increasing the susceptibility of the bird to potential IBDV field exposure (123, 266). Therefore, in order to continue protection of broiler chickens against IBDV, some producers follow an active immunization program of broiler flocks with MLVs and recombinant HVT (rHVT-IBD) (269). Here, we have conducted studies to assess the efficacy of above mentioned broiler vaccines against vIBDV in broilers. Previous studies have compared the efficacy of these broiler vaccines against vIBDV by challenging broilers at 20-30 days of age where vIBDV infection is likely at a chronic stage or at the end of birds' susceptibility to vIBDV to cause a subclinical disease (277, 279, 280, 278, 281). Chickens are commonly processed around 35-45 days of age in the Canadian broiler chicken industry hence; diseases that occur at a young age are of great economic importance. Early age infection with IBDV results in long-lasting immunosuppression and profound economic losses. Therefore, our study primarily focussed on young age infection with vIBDV (6 days of age) and compared the protective effects of two commercial broiler vaccines frequently used in Canadian broiler chicken industry. In spite of

vaccination, birds showed bursal atrophy and lymphoid depletion following vIBDV-SK09 challenge. When two vaccines were compared, we found that MLV was able to delay vIBDV experimental infection. Our work also revealed a previously unknown phenomenon vis-à-vis HVT-IBDV vaccine, showing HVT-IBDV induces immunosuppression that helps the vIBDV replication (283). Thus HVT-IBDV vaccine may lead to decreased immune function against vIBDV infection, facilitating substantial viral growth in the host, consequently leading to early bursal damage. However, neither vaccines provided complete protection against early age infection with vIBDV-SK09 as indicated by severe bursal damage at 35 days age. Our data provide novel information regarding current broiler vaccines and highlight the need for suitable vaccine(s) against vIBDV in the broiler chicken industry in Canada.

The last chapter in this thesis explains the potential of using Canadian field isolates of vIBDV as vaccine candidates. Broiler breeder parents were vaccinated once with the bursal homogenate of live vIBDV-(SK09, SK10, SK11, SK12 or SK13) at 13 weeks of age. Broilers breeders had a high level of antibodies against IBDV 3 weeks after the vaccination and antibody level remained maintained at the same level until 45 weeks of age. The progeny of broiler breeders vaccinated with SK09 or SK10 were protected against vIBDV-SK 09 challenge at a significant level compared to broiler chickens from their parents not vaccinated against IBDV or commercial broilers from their parents vaccinated with conventional IBDV vaccines. We observed a strong immunoprotection of broilers against vIBDV-SK09 even when their parents were give single vIBDV-SK09 vaccine. It is worth mentioning that the broiler chicken challenged with IBDV strain SK09 were protected if broiler breeders received vIBDV-SK10 as a vaccine antigen. This cross protection is likely associated with sequence similarity of SK09 to SK10 (97.5 %).

In conclusion, we have demonstrated that current broiler breeder and broiler vaccines are not effective against vIBDV circulating in the Canadian broiler chicken industry. Because of this Canadian broiler chicken industry is prone to immunosuppression and secondary infections, incurring heavy economic losses (239). We have also demonstrated the possibility of using vIBDV-SK09, a representative strain currently circulating in the Canadian broiler chicken industry, as vaccine candidate against vIBDV infection to prevent IBD and ensuing profound immunosuppression in the poultry industry.

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